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# *EXPLORATION OF THE INTERRELATIONSHIP OF SEROTONIN WITH THE EPINEPHRINES AND HISTAMINE**

By D. W. Woolley†

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*Communicated May 2, 1960*

The purposes of this paper are (*a*) to call attention to evidence of direct and indirect biological relationships between pairs of neurohormones, and (*b*) to present experimental findings which indicate that some of these interactions may be the result of a direct competition of the hormones concerned for a specific receptor.

Many bits of evidence have accumulated which taken together indicate a subtle interplay of these hormones. The individual pieces of evidence are frequently vague and if this were not so there would be no need for this paper. When seen together, however, as is intended in this paper, they are highly suggestive. Let us therefore examine these bits of evidence for several pairs of hormones. The hormones in question are those which are known to cause smooth muscles to contract and to participate in the functioning of nerves. The best-known ones are serotonin, epinephrine, norepinephrine, histamine, and acetylcholine.

*Serotonin and Epinephrine or Norepinephrine.*—Erspamer[1] first described how the contracting action of serotonin on the isolated rat uterus was antagonized by epinephrine, and to a lesser extent by norepinephrine. This observation has been confirmed and extended by others.[2–4] Antagonism is, however, not the only sign of interplay of serotonin with the epinephrines. On some tissues antagonism is indeed seen, but in other tissues one finds instead a potentiation or even synergism. This point has been summarized by Gordon *et al.*[4] Such evidences of interplay have been noted mainly with smooth muscles, but have also been obtained in preparations involving nerve action directly or indirectly. In addition, much recent theorizing prompted by findings with reserpine in relation to serotonin and norepinephrine[5–7] also has turned thoughts towards such an interplay of these hormones in the central nervous system. Reserpine depletes nerves and certain other tissues of serotonin and norepinephrine, and the suggestion has been made that its effects on nerve function and on animal behavior are related to this depletion.

*Acetylcholine and Epinephrine.*—The antagonism of epinephrine to acetylcholine in the rat uterus and in other tissues has been known for a long time and forms the basis of several methods for the bioassay of epinephrine and of norepinephrine.

*Acetylcholine and Serotonin.*—The interplay of acetylcholine and serotonin (i.e., the antagonism or the potentiation) has been seen for the most part indirectly and

vaguely. Thus, antiacetylcholine drugs such as atropine which do not interfere with serotonin in many tissue preparations have been found to interfere with it in some.[8, 9] Herxheimer[8] made such observations in guinea pigs subjected to shock caused by inhalation of aerosols of serotonin, or to anaphylactic shock. Woolley[10] showed the synergistic effects of acetylcholine and serotonin given intracerebrally in overcoming the behavioral changes induced in mice with lysergic acid diethylamide (LSD). Turning from these examples of synergism and potentiation to antagonism we see that the antagonisms of the actions of serotonin and of acetylcholine in their effects on the heart[11] and on the control of blood pressure[12] have been observed. These antagonisms are probably indirect. They probably do not involve a single receptor but rather express the physiological antagonism of certain sympathetic (serotonergic) and parasympathetic (cholinergic) nerves. Understanding here is rudimentary, and the existence of a problem is just now being discerned. However, we do begin to see that sometimes these two hormones act together and sometimes they act against each other in the control of some gross physiological process.

*Histamine and Serotonin or Epinephrine.*—With histamine and serotonin, or with histamine and the epinephrines, very little evidence for interplay is available, but there is some. None of this is very direct. Thus, the participation of both histamine and serotonin in allergic responses first clearly recognized by Herxheimer[8] is of interest partly because in mice serotonin seems to play the dominant role whereas in guinea pigs it seems to be histamine. In either species, however, both substances seem to be involved. There is also the evidence obtained by the use of drugs to suggest some close connection between the biological actions of histamine, serotonin, and the epinephrines. Thus, chlorpromazine which was developed during the routine screening of compounds for antihistamine action, is not only a weak antihistamine (and a structural analog of that hormone), but also a powerful antagonist of serotonin[13] and of epinephrine[14] in suitable tissues. Several other pharmacological agents have likewise been found to interfere with the actions of each of these hormones even though structurally analogous only to one. Such findings suggest that the actions of these several hormones are interconnected and that interference with the action of one results in some impairment of the actions of the others.

*Epilogue.*—Taken together such evidence suggests that these hormones which act on smooth muscles and on nerves probably are interconnected in their biological effects. The functioning of these hormones seems to touch at many points and influencing one may influence some of the others.

In thinking about such instances of interplay among these hormones we see that there are some which are direct and some which are indirect. Direct interplay means that the two hormones which show it are acting on a single set of chemical reactions in a given tissue. Thus, if serotonin and norepinephrine both reacted with a single kind of receptor in a smooth muscle the interplay would be direct whether or not the result of the interplay was antagonism or synergism. Indirect interplay, on the other hand, means that the one hormone actuates a function in one tissue which either helps or hinders a related function in another tissue. Thus, if acetylcholine from the vagus nerve slows the heart and serotonin from the stellate ganglion stimulates the heart by acting on an independent mechanism in

that organ this would be indirect antagonism. Both direct and indirect interplays are discernible among the hormones being discussed.

*Working Hypothesis.*—One plausible working hypothesis to explain the direct interplay of two of these hormones, let us say serotonin and norepinephrine, is that in those tissues where antagonism is found, one hormone combines with and blocks the receptor for the other. Thus, norepinephrine might be pictured as combining with the serotonin receptor just as an antimetabolite of serotonin does, but, because norepinephrine is not identical to serotonin, the norepinephrine cannot activate the receptor. As a result one finds no serotonin-like action of norepinephrine on this particular tissue, but instead, an antagonism of the action of serotonin. In other tissues it might be that the receptors are not so exacting, so that the norepinephrine might still activate the serotonin receptor mechanism. Or, in these other tissues there may be both serotonin and norepinephrine receptors which would explain the positive response to either hormone. In these tissues, then, one would find only potentiation. Such an hypothesis would account for the data with pairs of hormones which have a structural resemblance such as serotonin and the epinephrines. These might be called cases of direct interplay because they involve just one receptor and one function. However, many instances of indirect relationship probably also exist, such as, for example, the antagonism mentioned above of acetylcholine and serotonin on the heart. Probably both direct and indirect mechanisms must be invoked to understand all of the types of relationship for these "neuromuscular" hormones.

The present work has sought to investigate the antagonism between serotonin and the epinephrines on isolated rat uterus, and to study the idea that the antagonism is exerted through the serotonin receptor mechanism (direct interplay). In addition, an antagonism between serotonin and histamine on this same tissue has been found, and when studied similarly, has been shown to be much less dependent on the serotonin receptor (indirect interplay).

These investigations were made possible by the finding that the mechanism of action of serotonin on rat uterus is probably to cause the transport of calcium ions across the muscle cell membrane.[15, 16] If serotonin does cause the muscle to contract because it carries calcium ions into the cell to activate the actomyosin-adenosinetriphosphate system, then a specific antagonist such as an antimetabolite of serotonin should block this transport mechanism. The contractile machinery (the actomyosin-ATP system) and the energy-yielding processes which generate the ATP should not be influenced by such an antagonism. All that is lacking to set the machinery in motion is calcium ions. Consequently, if calcium ions were to be brought into the cell by a mechanism independent of serotonin, then contraction should take place despite the fact that the serotonin mechanism for transporting calcium ions has been blocked. One way to get calcium ions into the cell without the intervention of serotonin or other hormones is to increase the concentration of these ions outside the cell. Enough is then carried in by leakage in the lipoidal membrane, or by pinocytosis, to activate the contractile machinery. Woolley[17] has shown that when a specific antimetabolite of serotonin is used to prevent the muscle from contracting when serotonin is applied, contraction can still be elicited merely by increasing the calcium ion concentration in the surrounding fluid. However, if some other inhibitor of contraction is applied which does not

specifically block serotonin, then application of more calcium ions will not cause contraction. This technique has now been used to study the antagonism to the contractile action of serotonin caused by epinephrine, norepinephrine, and histamine in rat uterus. If the antagonism could be overcome with excess calcium ions, it would point to the serotonin receptor mechanism for transporting calcium ions as the site of the antagonism. If calcium ions did not overcome the antagonism, some less direct relationship than that involving competition for the serotonin receptor would be indicated.

*Materials.*—The low-calcium Ringer solution was that described by Woolley.[15] Serotonin or 5-hydroxytryptamine-creatinine sulfate, DL-norepinephrine hydrochloride, L-epinephrine, and histamine dihydrochloride were fresh, pure samples. The weights given were of the salts not the free bases (except for epinephrine). Solutions of these hormones were made by use of the low-calcium Ringer's solution, and were always freshly made. The pH of all solutions was adjusted to that of the Ringer's solution in order to avoid the interference caused by acid in the contraction of muscles brought about by serotonin.[17]

*Methods.*—The horn of a uterus from an estrogenized rat, prepared as described earlier,[15] was suspended in oxygenated low-calcium Ringer's solution at 30°. Responses to graded doses of serotonin were determined and recorded on a kymograph. After the dose of serotonin had been determined which would give nearly maximal contraction reproducibly, the tissue was washed and norepinephrine was applied. Without washing, the predetermined dose of serotonin was again added, and the response was measured. The amount of serotonin required was 0.005 to 0.02 $\mu$g per ml, and the amount of norepinephrine for the first trial was 0.001 $\mu$g per ml. The tissue was washed and increasing graded doses of norepinephrine were then each similarly assayed until complete inhibition of the serotonin-induced contraction was obtained. In the same way, epinephrine, acetylcholine, and histamine were tested for antiserotonin activity.

Sufficient inhibitor (norepinephrine, epinephrine, or histamine) to cause almost complete antagonism of the serotonin-induced contraction was applied to the tissue, and then, without washing, calcium chloride was added. The amount of calcium chloride required to cause maximal contraction in the absence of the hormones had been established for each tissue specimen earlier, and this was the amount used. If no contraction occurred with calcium chloride in the presence of the hormone, the dose of calcium was doubled in a second trial. Similar experiments were performed in which the specific antimetabolite of serotonin BAS or 1-benzyl-2-methyl-5-methoxytryptamine was used instead of the hormonal inhibitors.[18]

Results of all experiments were confirmed at least three times on tissues from different rats.

*Results.*—The ability of norepinephrine to prevent the contraction of the uterus caused by serotonin was overcome by calcium ions. In other words, calcium ions still made the muscle contract in the presence of norepinephrine, even though serotonin would not.

The same thing was true when epinephrine was the inhibitor of the action of serotonin. Similarly, the specific antimetabolite of serotonin (BAS) behaved as did the epinephrines in this respect. Calcium ions still caused the muscle to contract in the presence of epinephrine or of BAS, even though serotonin did not.

TABLE 1

ANTAGONISM TO SEROTONIN OF EPINEPHRINE AND NOREPINEPHRINE AND ITS REVERSAL WITH CALCIUM CHLORIDE

| Serotonin (μg/ml) | $CaCl_2$* (μg/ml) | Epinephrine (μg/ml) | Norepinephrine (μg/ml) | Contraction (cm) |
|---|---|---|---|---|
| 0.005 | 0 | 0 | 0.00 | 2.7 |
| 0 | 0 | 0 | 0.10 | 0.0 |
| 0.005 | 0 | 0 | 0.01 | 1.6 |
| 0.005 | 0 | 0 | 0.03 | 1.0 |
| 0.005 | 0 | 0 | 0.10 | 0.2 |
| 0.005 | 0 | 0 | 0.00 | 0.9† |
| 0.020 | 0 | 0 | 0.00 | 3.5† |
| 0 | 200 | 0 | 0.00 | 2.5 |
| 0 | 200 | 0 | 0.10 | 2.3 |
| | | New uterus | | |
| 0.01 | 0 | 0 | 0 | 3.5 |
| 0.01 | 0 | 0.001 | 0 | 0.2 |
| 0.01 | 0 | 0 | 0 | 1.1 |
| 0.02 | 0 | 0 | 0 | 4.5† |
| 0.02 | 0 | 0.001 | 0 | 5.0 |
| 0.02 | 0 | 0.003 | 0 | 4.2 |
| 0.02 | 0 | 0.010 | 0 | 0.2 |
| 0.02 | 0 | 0 | 0 | 0.4† |
| 0.04 | 0 | 0 | 0 | 3.7† |
| 0 | 400 | 0 | 0 | 2.5 |
| 0 | 400 | 0.01 | 0 | 2.2 |

* $CaCl_2$ in addition to that in the Ringer's solution which supplied 20 μg/ml.
† Contractions elicited on retesting the tissue after the treatments preceding in the table.

Data to illustrate these points are given in Table 1. The findings with BAS were the same as those already published.[17]

Histamine, like the epinephrines, was also an antagonist to the action of serotonin, as can be seen from the data in Table 2. However, when histamine was used as the inhibitor, calcium ions would no longer cause the muscle to contract.

Comparisons were made on tissues from the same rat of the relative potencies of norepinephrine, epinephrine, and histamine, as antagonists of a given dose of serotonin. Epinephrine was the most active and histamine was the least. Epinephrine was somewhat more potent than the antimetabolite of serotonin known as BAS, a compound which has been outstanding for high potency as an antagonist of serotonin.[18, 19]

Epinephrine, norepinephrine, and histamine each caused irreversible loss of

TABLE 2

ANTAGONISM TO SEROTONIN OF HISTAMINE AND THE EFFECT ON IT OF CALCIUM CHLORIDE

| Serotonin (μg/ml) | $CaCl_2$* (μg/ml) | Histamine (μg/ml) | Contraction (cm) |
|---|---|---|---|
| 0.01 | 0 | 0 | 0.4 |
| 0.02 | 0 | 0 | 2.4 |
| 0 | 0 | 1.0 | 0.0 |
| 0 | 300 | 0 | 3.1 |
| 0.02 | 0 | 0.1 | 0.6 |
| 0.02 | 0 | 0 | 0.3† |
| 0.03 | 0 | 0 | 3.1† |
| 0.03 | 0 | 0.3 | 0.3 |
| 0.03 | 0 | 0 | 1.2† |
| 0 | 300 | 0.3 | 0.0 |
| 0 | 300 | 0 | 3.1† |
| 0.03 | 0 | 0 | 2.0 |

* $CaCl_2$ in addition to that in the Ringer's solution which supplied 20 μg/ml.
† Contractions elicited on retesting the tissue after treatment with histamine and thorough washing, or after the treatments preceding in the table.

sensitivity of the tissue to serotonin. The data of Tables 1 and 2 will show that when enough of one of these hormones had been used to bring about suppression of the serotonin-induced contraction no amount of washing would restore the tissue to its original sensitivity. Furthermore, although larger amounts of serotonin were still able to cause contraction, the concentration of inhibitory hormone required to antagonize this effect was likewise increased. The usual finding was that loss of sensitivity to serotonin was not unlimited because eventually a point was reached beyond which epinephrine or norepinephrine ceased to bring about further loss in sensitivity to serotonin.

*Discussion.*—The present results suggest that epinephrine and norepinephrine interfere with the action of serotonin on rat uterus in a direct rather than a remote way. These results are compatible with the idea that the epinephrines may react with the serotonin receptors of the uterus, bringing about failure of these receptors to carry out their normal function, which may be to combine with serotonin plus calcium ions and to transport these ions into the cell in the manner previously outlined.[15] The present results show that when calcium ions were forced into the muscle cell by means other than that involving serotonin, i.e., by increase in the concentration outside of the cell, the inhibited muscle was quite capable of contraction.

By contrast, the antagonism exerted by histamine appeared to be somewhat less direct. With histamine, increased calcium ion in the extracellular fluid did not cause contraction. This might suggest that histamine blocked the action of serotonin, not by stoppage of the entry of calcium ions, but rather by some interference with those mechanisms inside of the cell by means of which adenosinetriphosphate is generated and caused to combine with actomyosin plus calcium ions. However, the biochemical events by means of which ion transport through the cell membrane takes place are so poorly understood that the action of the histamine might still be to block some event at the membrane on which calcium ion entry depends. Histamine might, for example, stop pinocytosis or close up the "holes" in the lipoidal membrane through which excess calcium ions in the exterior medium are thought by some to "leak" into the cell. In any event, the technique of applying calcium ions to overcome the antagonism of these hormones to serotonin has revealed that the antiserotonin action of the epinephrines is similar, but different from that of histamine. The technique has shown further that the antiserotonin action of the epinephrines is similar to that of a recognized, specific antimetabolite of serotonin, viz., BAS.

*Summary.*—Attention has been called to the interplay in biological actions of a group of hormones which cause smooth muscles to contract, and which function in nerves. These hormones include serotonin, norepinephrine, epinephrine, histamine, and acetylcholine. Sometimes the interplay is expressed as potentiation or synergism, and sometimes as antagonism. The antagonism of norepinephrine and epinephrine to serotonin on rat uterus was studied further, and the antagonism of histamine to serotonin on this tissue was described. Calcium chloride erased the antagonism to serotonin of the two epinephrines but not that of histamine. In this respect, the two epinephrines behaved as did a specific antimetabolite of serotonin, viz., 1-benzyl-2-methyl-5-methoxytryptamine (BAS). The findings were seen to be compatible with the previous evidence that serotonin acts on tissues

by causing transport of calcium into the cells and that the antiserotonin activity of the epinephrines may be because they combine with the serotonin receptor and block it as an antimetabolite would do. The antagonism of histamine seemed less direct.

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† With the technical assistance of N. K. Campbell.

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## *A HIGH-ENERGY INTERMEDIATE OF OXIDATIVE PHOSPHORYLATION**

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*Communicated by David M. Bonner, May 2, 1960*

Previous reports from this laboratory have described an enzyme system from the bacterium *Alcaligenes faecalis* which couples the formation of ATP† to the oxidation of DPNH. The complex contains a particulate DPNH oxidase, a soluble heat labile fraction, and a polynucleotide of the RNA type. One of the functions of this polynucleotide, in conjunction with magnesium, is to bind the soluble heat labile factor to the particles.[1–5]

The purpose of this communication is to show that the enzyme system is dissociated into particulate and soluble components by incubation with DPNH, but not with DPN. The soluble fraction thus eluted produces net ATP synthesis when incubated with Pi and ADP. It is therefore proposed that this compound is a high energy intermediate of oxidative phosphorylation. Two techniques were used for measuring phosphorylation by this intermediate. The first used $P^{32}$ incorporation into organic phosphate, and the second measured ATP formed with the firefly assay system.

*Methods.*—The bacteria were grown, washed, and sonically disrupted as pre-

viously described.[1, 2] The media was fortified with salts[6] and 2 mg/L of calcium pantothenate. The oxidative phosphorylation complex was precipitated by centrifugation in the presence of $MgCl_2$ at 100,000 $\times$ $g$ for 20 min at approximately 0°. The pellet was then resuspended and reprecipitated to yield washed phosphorylating particles.[5] Hexokinase was made by the method of Colowick and was twice recrystallized.[7]

*Experimental.—Dissociation of enzyme complex with DPNH:* Washed phosphorylating particles containing approximately 10–15 mg of protein and 8–10 per cent nucleic acid were incubated in a total volume of 2.5 ml at 24° for 3–5 min in the standard preincubation mixture containing 12 $\mu$moles of $MgCl_2$, 5.9 $\mu$moles of KCl, and either no pyridine nucleotide or 3 $\mu$moles of DPN or DPNH as indicated. The reaction mixtures were then rechilled and recentrifuged. The supernatant solutions were saved and the pellets were resuspended in 0.01 $M$ KCl. The pellets were then tested for oxidative phosphorylation with DPNH as substrate, and the supernatant solutions were tested for their ability to esterify Pi without added substrate.

The pellets were tested for oxidative phosphorylation in a mixture of glycylglycine and phosphate buffers pH 7.4 with the final Pi concentration 1 $\mu$mole/ml, $MgCl_2$, ATP or ADP, hexokinase, glucose, and $P^{32}$. DPNH was used as substrate and a control vessel contained an equal amount of DPN.[2] The difference in phosphorylation observed between the experimental and control vessels is the value reported as oxidative phosphorylation. Incorporation of $P^{32}$ into the organic fraction was estimated by a modification of the method of Berenblum and Chain,[8–10] which depends on extracting the molybdic acid complex of Pi with isobutanol benzene. All samples were timed with a thin window gas flow counter for 1,000 counts and were corrected for background.

Incorporation of phosphate by the supernatant solutions was measured in a similar medium, except that the glycylglycine and phosphate concentrations were just one-half the concentrations used in testing the pellets, and in most cases ADP was the acceptor without added hexokinase or glucose. No substrate was added to the supernatant solutions as the experiments were designed to examine the question of whether or not a high energy intermediate exists in the supernatant solution obtained from the preincubation with DPNH. If it does, then $P^{32}$ would be incorporated into the organic fraction without the addition of an oxidizable substrate. The supernatant solutions from preincubation with DPNH and DPN will be referred to as $S^{DPNH}$ and $S^{DPN}$.

*Effect of preincubation with DPNH:* Results of typical experiments are shown in Table 1. Preincubation with DPNH, but not with DPN, caused a marked inhibition of oxidative phosphorylation in the pellets as seen in lines 1 and 3. It is also evident that $S^{DPNH}$ catalyzed esterification of a large amount of Pi in comparison with the control supernatant solutions from preincubations with DPN or no pyridine nucleotide. This is shown in lines 2 and 4. When Pi, hexokinase, ADP, and glucose are added to the standard preincubation mixture, the inhibition of oxidative phosphorylation in the pellets caused by preincubation with DPNH is much reduced. Associated with this, there is virtually no phosphorylation catalyzed by the supernatant solution. In other experiments inclusion of dinitrophenol ($2 \times 10^{-3}$ $M$) and Pi, or of arsenate ($1 \times 10^{-3}$ $M$) in the preincubation

mixture, produced similar though less consistent effects in reducing the inhibition of oxidative phosphorylation in the pellets preincubated with DPNH.

The second half of Table 1 shows that the inhibition caused by preincubation with DPNH on oxidative phosphorylation catalyzed by the pellet can be largely reversed by adding $S^{DPNH}$ back to the pellet several minutes before testing.

These results are compatible with the hypothesis that, during oxidation of DPNH by the enzyme system, a soluble component is dissociated in the high energy state. This factor is required for oxidative phosphorylation, but cannot become reassociated with the particles until the high energy bond is cleaved by phosphate. Dinitrophenol and arsenate presumably play similar roles by accelerating hydrolysis of the high energy intermediate, thus freeing it for recombination with particles. There are, of course, a number of other plausible explanations for the observations which will be discussed below.

*Relationship to oxidative phosphorylation:* During the preincubation of the enzyme complex with DPNH there is a marked shift to an alkaline pH since the system is weakly buffered. Addition of glycylglycine at pH 6.0, 7.4, and 8.0 either prevents or markedly decreases the effect of preincubation with DPNH. This suggests that the effect observed may be a pH effect alone and not specifically related to oxidative phosphorylation. This does not seem to be the case for two reasons. First, $S^{DPNH}$ has a marked stimulatory effect on oxidative phosphorylation catalyzed by particles preincubated with DPNH as shown in the second part of Table 1. In addition $S^{DPNH}$ has been shown to replace the heat labile factor in the ammonium sulfate separated system and conversely heat labile factor from the ammonium sulfate fractionation restores phosphorylation to particles preincubated with DPNH. Secondly, preliminary experiments by Dr. G. Hovenkamp have shown that there is a consistent difference in phosphorylation by $S^{DPNH}$ and $S^{DPN}$ in strongly buffered systems at higher pHs.

TABLE 1

THE EFFECT OF PREINCUBATION WITH DPNH ON AN ENZYME COMPLEX WHICH CATALYZES OXIDATIVE PHOSPHORYLATION

| Expt. | Additions to standard preincubation mixture | Fraction tested | Phosphorylation in millimicromoles after preincubation with: No pyr. nucl. | DPN | DPNH | % Inhib. due to DPNH |
|---|---|---|---|---|---|---|
| 1 | None | Pellet | 111 | 91 | 15 | 86 |
| 1 | None | Supern. Sol. | 1.7 | 2.0 | 13.4 | .. |
| 2 | None | Pellet | 161 | 110 | 37 | 77 |
| 2 | None | Supern. Sol. | 1.0 | 1.6 | 7.8 | .. |
| 2 | Pi, Hexo, ADP, Gluc. | Pellet | 119 | 115 | 72 | 40 |
| 2 | Pi, Hexo, ADP, Gluc. | Supern. Sol. | 1.0 | 0.9 | 1.8 | .. |
| 3 | None | Pellet | ... | 87 | 5 | 94 |
| 3 | Pi 0.001 *M* | Pellet | ... | 69 | 19 | 72 |
| 3 | Pi, Hexo, ADP, Gluc. | Pellet | ... | 50 | 24 | 52 |

Reconstitution of Pellet Activity

| | Additions to pellet | No pyr. nucl. | DPN | DPNH | % Inhib. due to DPNH |
|---|---|---|---|---|---|
| 4 | None | 101 | 80 | 27 | 73 |
| 4 | Sup. of no pyr. nucl. | ... | .. | 37 | 63 |
| 4 | Sup. of DPNH | ... | .. | 73 | 27 |

Perhaps the most obvious alternative possibility to the hypothesis outlined is that an enzyme that catalyzes a Pi-ATP exchange reaction, has been eluted from the particles.[11-14] Polynucleotide phosphorylase could also lead to $P^{32}$ uptake

if it was present in $S^{DPNH}$.[15] Finally, soluble oxidative phosphorylation remains a remote possibility since $S^{DPNH}$ contains significant amounts of unoxidized DPNH. The experiments described below were designed to evaluate the hypothesis of a high energy intermediate in $S^{DPNH}$ and the alternative explanations outlined.

*Proportionality:* If a finite amount of a high energy compound exists in the $S^{DPNH}$, then the amount of phosphorylation observed at completion of the reaction should be proportional to the amount of $S^{DPNH}$ used, while if the findings are to be explained on the basis of a Pi-ATP exchange reaction, the final amount of $P^{32}$

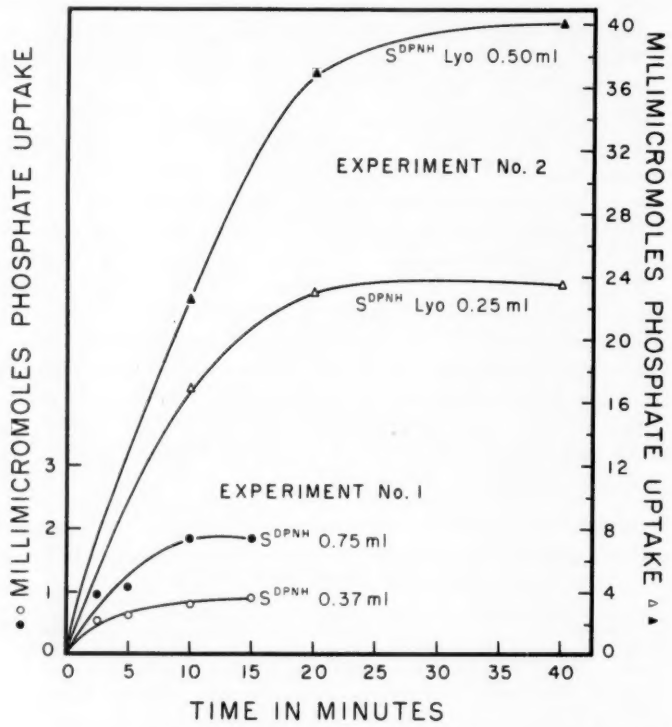


FIG. 1.—Proportionality between amount of $S^{DPNH}$ used and amount of phosphorylation observed. For experiment 1 use left scale and for experiment 2 use right scale for phosphorylation.

incorporated should be independent of the amount of $S^{DPNH}$ used. Figure 1 shows the results of two experiments bearing on this point. In the first experiment fresh $S^{DPNH}$ was used, and the figures plotted represent the phosphorylations observed with the boiled blanks subtracted. In experiment 2, $S^{DPNH}$ was concentrated by lyophilization and the reaction was run in the presence of 0.05 *M* NaF, which has a marked stimulatory effect on the phosphorylation observed from $S^{DPNH}$ (*vide infra*). These results indicate a reasonable proportionality between the amount of phosphate esterified at completion of the reaction and the amount of $S^{DPNH}$ used.

TABLE 2

THE EFFECT OF PREINCUBATION OF $S^{DPNH}$ WITH SALTS BEFORE $P^{32}$ ADDITION (PHOSPHATE ESTERIFICATION IN MILLIμMOLES)

| Preincubation with | | | Phosphorylation by $S^{DPNH}$ | Phosphorylation $S^{DPN}$ No Preincubation | Phosphorylation $S^{DPNH}$ boiled No Preincubation |
|---|---|---|---|---|---|
| Glycylglycine pH 7.4, $2.5 \times 10^{-2}$ *M*, | $MgCl_2$ | 6 μM/ml | 7.24 | 0.2 | 0.4 |
| KCl $1 \times 10^{-3}$ *M*, | " | " | 5.80 | ... | ... |
| $KHPO_4$ pH 7.4, $5 \times 10^{-4}$ *M*, | " | " | 1.07 | ... | ... |
| $KHPO_4$ pH 7.4, $5 \times 10^{-5}$ *M*, | " | " | 2.77 | ... | ... |
| $KHPO_4$ pH 7.4, $5 \times 10^{-6}$ *M*, | " | " | 5.51 | ... | ... |
| $Na_2HAsO_4$ pH 7.4, $5 \times 10^{-4}$ *M*, | " | " | 2.12 | ... | ... |
| $Na_2HAsO_4$ pH 7.4, $5 \times 10^{-5}$ *M*, | " | " | 4.48 | ... | ... |
| $Na_2SO_4$ pH 7.4, $5 \times 10^{-4}$ *M*, | " | " | 4.37 | ... | ... |

*Discharge of intermediate:* If a high energy intermediate exists in $S^{DPNH}$, then it should be phosphorylized by preincubation with cold phosphate and, on later addition of $P^{32}$ and ADP, little or no $P^{32}$ should be taken up since all the high energy intermediate would be combined with cold phosphate and none would be left for combination with $P^{32}$. $S^{DPNH}$ phosphorylized with cold Pi is referred to as Pi-discharged $S^{DPNH}$. Similarly, preincubation with arsenate would be expected to interfere with subsequent phosphorylation. To examine this point, fresh $S^{DPNH}$ was incubated in the presence of $MgCl_2$ with various concentrations of cold phosphate, arsenate and, as controls with sulfate, glycylglycine, or KCl, for 5 min at room temperature. These preincubation mixtures were then poured into tubes containing $P^{32}$, ADP, and appropriate additions to make the final glycylglycine concentration 0.025 *M*, and the phosphate 0.0005 *M*. Table 2 shows an experiment representative of many similar ones. Preincubation of $S^{DPNH}$ with glycylglycine (shown to have little effect in previous experiments) or KCl produces comparatively small effects on the amount of phosphorylation, while phosphate and arsenate do produce more marked effects dependent on their concentrations. Sulfate produces a comparatively small effect. These results are again compatible with the hypothesis of a high energy intermediate and provide further evidence against a Pi-ATP exchange.

*Specificity of nucleotide acceptor:* In order to get more definite information about a possible Pi-ATP exchange reaction, the effectiveness of ATP and AMP were compared with ADP as acceptors of phosphate in the presence of $S^{DPNH}$. These

TABLE 3

THE EFFECT OF PHOSPHATE, ADP, AMP, AND ATP ON PHOSPHORYLATION BY $S^{DPNH}$ (PHOSPHORYLATION IN MILLIμMOLES)

| Acceptor | Phosphorylation after preincubation with No pyridine nucleotide | DPN | DPNH |
|---|---|---|---|
| ADP 0.10 μM no hexokinase, glucose | 1.74 | 1.46 | 6.16 |
| ADP 0.50 μMole | ... | ... | 4.98 |
| ADP 0.05 μMole | ... | ... | 3.79 |
| ADP 0.005 μMole | ... | ... | 2.23 |
| ATP 0.50 μMole | ... | ... | 2.47 |
| ATP 0.05 μMole | ... | ... | 2.37 |
| ATP 0.005 μMole | ... | ... | 2.32 |
| AMP 0.10 μMole | ... | ... | 2.31 |
| ADP 0.1 μMole, hexokinase glucose | 1.37 | 1.46 | 9.25 |
| No glycylglycine | ... | ... | 9.05 |
| No $PO_4$ | ... | ... | 0.014 |
| No hexokinase | ... | ... | 9.10 |
| NO ADP | ... | ... | 2.57 |

experiments were run in the absence of hexokinase and glucose. The results in Table 3 are representative of several similar experiments and show that ADP is a more effective acceptor from $S^{DPNH}$ than either ATP or AMP. The second experiment (shown below the line in the table) indicates that some phosphorylation takes place without added acceptor, and that the phosphorylation with added ATP or AMP is not significantly above this basal level which is presumably the result of endogenous acceptor. These facts do not fit a Pi-ATP exchange hypothesis or any synthetic reaction requiring ATP which might simulate such an exchange. The very marked stimulation of phosphorylation produced by addition of ADP would rule out the possibility that the $P^{32}$ incorporation was dependent on phosphorolysis of a polynucleotide, although the possibility might exist that there was an exchange going on with polynucleotide synthesis and breakdown dependent

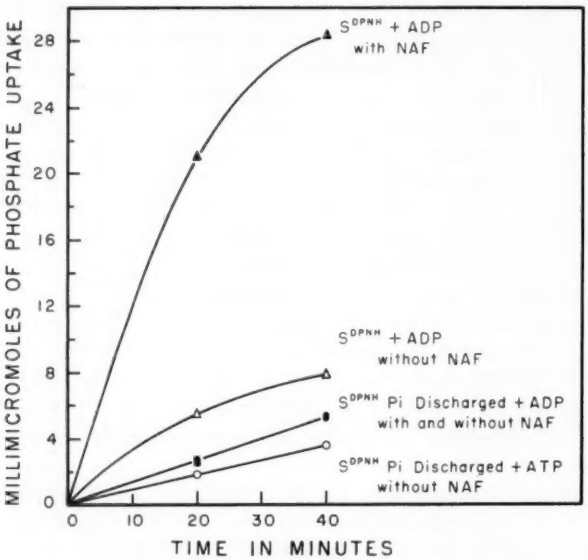


FIG. 2.—The effect of NaF on phosphorylation by $S^{DPNH}$.

on mononucleotide and a polynucleotide primer. This possible role of polynucleotide phosphorylase will be discussed later.

*Effect of inhibitors:* The rather unlikely possibility that $S^{DPNH}$ contains a soluble oxidative phosphorylation system is made even more remote by the effects of inhibitors. Dinitrophenol in a concentration of $2 \times 10^{-3}$ *M* causes an inhibition of from 60 to 90 per cent in phosphorylation linked to DPNH oxidation in the particulate system, but produced only 3–5 per cent inhibition in phosphorylation with $S^{DPNH}$. Similarly, 0.05 *M* NaF caused an 87 per cent inhibition in oxidative phosphorylation catalyzed by the particulate system, while it produced a marked stimulation in the amount of phosphorylation from $S^{DPNH}$ as shown in Figure 2. It has no effect on the comparatively slow rate at which $P^{32}$ is incorporated by $S^{DPNH}$ which has been exposed to cold phosphate to cleave the high energy inter-

mediate before the addition of $P^{32}$ and ADP. This suggests that these are two unrelated reactions. The stimulatory effect of NaF on phosphorylation with $S^{DPNH}$ is presumably due in part at least to its effect on ATPase since there is a weak ATPase in $S^{DPNH}$ (100 milli$\mu$moles ATP hydrolyzed/ml/hr) which is partially inhibited by NaF. This was determined with hexokinase and glucose-6-phosphate dehydrogenase and TPN. Figure 2 also shows that the rate of phosphorylation using discharged $S^{DPNH}$ is slower when ATP is used as acceptor than with ADP. This again provides clear evidence against the exchange reaction hypothesis.

*Effect of polynucleotide, coenzyme concentrate, and boiled $S^{DPNH}$:* If the esterification of $P^{32}$ by $S^{DPNH}$ were dependent on a polynucleotide primer in the $S^{DPNH}$, then addition of polynucleotide or boiled $S^{DPNH}$ to Pi-discharged $S^{DPNH}$ would be

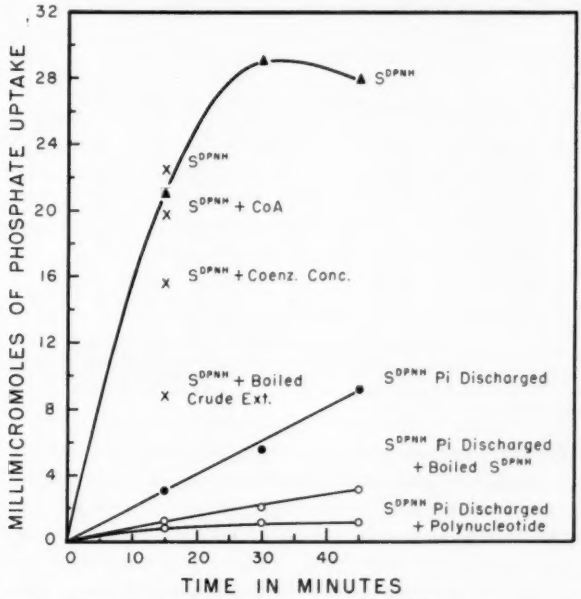


FIG. 3.—The effects of polynucleotide, boiled $S^{DPNH}$, and coenzymes on phosphorylation by $S^{DPNH}$.

expected to produce a stimulation of Pi esterification. Polynucleotide made from *Alcaligenes faecalis* washed particles,[2] which is required for oxidative phosphorylation by the complete system, produced a marked inhibition of $P^{32}$ esterification when added to phosphate-discharged $S^{DPNH}$. Boiled $S^{DPNH}$ had the same effect as shown in Figure 3. A crude liver coenzyme concentrate and boiled crude *Alcaligenes faecalis* extract, the latter also known to be active in oxidative phosphorylation, were both inhibitory when added to undischarged $S^{DPNH}$ in a separate experiment shown by crosses in Figure 3. Coenzyme A was slightly inhibitory. These experiments provide clear evidence that the incorporation of $P^{32}$ into the organic fraction by $S^{DPNH}$ cannot be explained by the action of polynucleotide phosphorylase in the supernatant solution.

In order to demonstrate conclusively that $S^{DPNH}$ contains a high energy intermediate, it was necessary to show net synthesis of ATP from ADP, Pi, and $S^{DPNH}$. The ATP assay with the firefly system seemed to be ideal for this since it is specific for ATP and is very sensitive. Dr. W. D. McElroy and Mrs. L. Geiger very kindly ran these assays.[16] It had already been found that $S^{DPNH}$ contained small amounts of adenylate kinase as shown in a test using hexokinase, glucose-6-phosphate dehydrogenase, and TPN to assay for ATP with ADP as substrate. Using the firefly system to assay for ATP formed, experimental vessels contained Pi, ADP, and $S^{DPNH}$. The control vessels had no Pi added and thus measured ATP formation from ADP catalyzed by adenylate kinase. This value was subtracted from the value found in the vessel containing Pi to give net ATP synthesis corrected for adenylate kinase. These values were compared with the values obtained with the same $S^{DPNH}$ solutions by the $P^{32}$ assay with the results shown in Table 4. It will

TABLE 4

PHOSPHORYLATION BY $S^{DPNH}$
COMPARISON OF $P^{32}$ UPTAKE AND ATP FORMATION

| | | 30 sec | | | 3 min | | |
|---|---|---|---|---|---|---|---|
| Expt. | Assay | Total | Control | Δ | Total | Control | Δ |
| 1 | $P^{32}$ | 13.0 | 1.0 | 12.0 | 30 | 3.5 | 26.5 |
| 1 | Firefly | 14.7 | 3.7 | 11.0 | 2.9 | 1.5 | 1.4 |
| 1 repeat | Firefly | 13.2 | 8.1 | 5.1 | 7.4 | 3.7 | 3.7 |
| 2 | $P^{32}$ | 6.4 | 0.9 | 5.5 | 19.0 | 1.7 | 17.3 |
| 2 | Firefly | 7.2 | 2.2 | 5.0 | 2.4 | ... | ... |
| 2 repeat | Firefly | 6.5 | 3.5 | 3.0 | ... | ... | ... |
| 2 No NaF | Firefly | 11.6 | 8.1 | 3.5 | ... | ... | ... |
| 3 | $P^{32}$ | 1.8 | 0.4 | 1.4 | 3.9 | 0.4 | 3.5 |
| 3 | Firefly | 1.1 | 0.5 | 0.6 | ... | ... | ... |

Results are expressed as millimicromoles of Pi uptake or ATP formation with the total figure observed, followed by the control to be subtracted and under Δ the difference. In the $P^{32}$ assay the control is phosphorylation by the Pi-discharged $S^{DPNH}$, and in the ATP assay it is phosphorylation without added Pi. All experiments run in presence of 0.05 *M* NaF except as indicated. Experiments 1 and 2 used lyophilized $S^{DPNH}$ while in Experiment 3 dialyzed lyophilized $S^{DPNH}$ was used.

be seen that there is good agreement between the results obtained in the two different ways in 3 separate groups of experiments at 30 sec. After this time the $P^{32}$ assay continues to show an increase while the ATP value determined by firefly assay falls. This means that ATP is being converted to something else by some unspecified pathway of ATP utilization. There is a good reason why the ATP values are somewhat lower than the $P^{32}$ figures even at 30 sec. This is explained by the fact that the lyophilized $S^{DPNH}$ used contains some Pi. In the second group of experiments the actual phosphate concentration was measured in the reaction mixtures. The experimental vessel contained 0.616 μmole of Pi/ml while the control contained 0.153 μmole/ml. Thus, the adenylate kinase control vessels probably measured some ATP formation from the intermediate and the corrected values are therefore somewhat low.

*Discussion.*—These experiments are divided into two groups by method. In the first group, $P^{32}$ incorporation into the organic fraction was used as the assay method, and in the second group, net synthesis of ATP was demonstrated using the firefly assay system. In order to show the accumulation of a high energy intermediate in the supernatant solution from the DPNH preincubation using $P^{32}$ as an assay system, one must rule out other known reactions by which $P^{32}$ can be incorporated into the organic phase. In this case these consist in $P^{32}$-ATP exchange

reactions, polynucleotide phosphorylase, and the unlikely one of soluble oxidative phosphorylation. The exchange reactions have been ruled out by showing that at completion of the reaction $P^{32}$ incorporation is proportional to the amount of supernatant solutions used, that cold phosphate incubation before addition of $P^{32}$ causes a marked inhibition of $P^{32}$ uptake, and that ADP is more active than ATP. The lack of activity of ATP may be somewhat puzzling when it is remembered that there is ATPase present in the supernatant solution from DPNH preincubation of the enzyme complex. The rate of ATP splitting is so slow, however, that at the end of the reaction some 25 milli$\mu$moles of ADP would be formed, while 50 milli$\mu$moles of ADP added at the beginning of the experiment causes only a small increase in phosphorylation over the basal level as shown in Table 3.

The possibility that polynucleotide phosphorylase action might explain the $P^{32}$ incorporation is ruled out by the fact that ADP causes a marked stimulation in $P^{32}$ incorporation and that polynucleotide made from *Alcaligenes faecalis* is strongly inhibitory. Soluble oxidative phosphorylation has been ruled out on the basis of studies with inhibitors.

While these results with $P^{32}$ incorporation are strongly suggestive that incubation of the enzyme system with DPNH produces an accumulation of an energy-rich intermediate of oxidative phosphorylation in the supernatant solution, they are not in themselves entirely conclusive, since they do not demonstrate net ATP synthesis. The experiments using the firefly assay demonstrate this clearly and, furthermore, are in close agreement at 30 sec with the results obtained with the $P^{32}$ assay. The subsequent decrease in ATP with time, and the continued rise in $P^{32}$ incorporation into the organic fraction, can only mean that the ATP formed by the reaction of Pi and ADP with the intermediate is being used by one or more of the many reactions by which the terminal phosphate of ATP is converted into other organic phosphate compounds in cells.

*Summary.*—(1) A soluble high energy intermediate of oxidative phosphorylation has been separated from phosphorylating particles during oxidation of DPNH.

(2) The intermediate has been assayed using $P^{32}$ as a measure of inorganic phosphate uptake and by direct measurement of ATP formation.

(3) Alternative explanations of the observations have been examined and rejected.

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* Contribution Number 277 from McCollum-Pratt Institute and the Department of Biology, Johns Hopkins University, Baltimore, Maryland.

† The following abbreviations will be used: ATP, ADP, and AMP for adenosine tri, di, and monophosphates, DPNH and DPN for reduced and oxidized diphosphopyridine nucleotide, Pi for inorganic phosphate, $S^{DPNH}$ for the supernatant solution recovered by centrifugation after preincubation of bacterial particles with DPNH, Hexo for hexokinase, Gluc. for glucose, DNP for 2,4 dinitrophenol.

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## *DIFFERENT ENZYMIC EXPRESSIONS OF MUTANTS OF HUMAN GLUCOSE-6-PHOSPHATE DEHYDROGENASE*

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The recent advances in understanding the specificity of genes on metabolic pathways[1] and the action of genes on molecular structure of protein[2] provide an impetus for the study of more complex interactions of genes at the level of biochemical differentiation and quantitative regulation of enzymes. These latter features are especially apparent in mutants of higher forms of life, where the deletion of an enzymic activity may be incomplete in regard to quantity, anatomical distribution, or timing.[3–5]

Particularly adaptable to such inquiries [4, 6, 7] has been the hereditary condition of humans, primaquine-sensitive hemolytic anemia,[8, 9] a disorder characterized by an 85–95 per cent reduction in activity of glucose-6-phosphate dehydrogenase (G-6-PD*)

$$\text{G-6-P} + \text{TPN} \rightleftharpoons \text{6-PG} + \text{TPNH}$$

in erythrocytes,[10] and by a tendency for these erythrocytes to undergo destruction when an affected individual takes primaquine or certain aromatic drugs.

Previous reports have dealt with a comparison of the lability, chromatographic behavior, and catalytic parameters of partially purified G-6-PD from normal (N) and primaquine-sensitive (S) erythrocytes.[4, 11, 12] No significant difference was found between the G-6-PD from both sources. Those findings are here supplemented with the studies of the erythrocytic G-6-PD from a Caucasian male infant with congenital non-spherocytic hemolytic anemia (H), a chronic hemolytic disorder which recently has been noticed also to be associated, in some pedigrees, with an almost complete absence in activity of erythrocytic G-6-PD.[13, 14] Because of the different racial stock from which such patients come and because of the more severe

and chronic symptoms, it has seemed reasonable to assume that congenital nonspherocytic hemolytic anemia represents a condition distinct from primaquine-sensitive hemolytic anemia. The genetic and clinical features of this particular case will be reported elsewhere.

*Materials and Methods.*—The G-6-PD was followed continuously, in a Beckman DU spectrophotometer, according to the method of Kornberg and Horecker,[15] as previously described.[12] The preliminary observations on hemolysates were made by using as a blank a cuvette containing the hemolysate and all components except G-6-P. Each assay on hemolysates contained the hemolysate from 1.3–2.5 $\mu L$ of packed erythrocytes in a 1.0 ml assay and necessitated the use of a photomultiplier. For the measurement of the Michaelis constant for TPN, greater sensitivity was obtained by using an exciting light of 360 m$\mu$ wavelength and measuring the fluorescence of TPNH at 450 m$\mu$ in an Aminco-Bowman spectrophotofluorometer.

By incorporating methods previously described,[4, 11] partial purification of the G-6-PD was achieved for these studies, as follows. Freshly drawn, heparinized, venous blood is centrifuged at 1,000–3,000 $g$ for 15 min and supernatant plasma withdrawn and discarded. The top layer of white cells is teased free of the hemolysate with the tip of a micropipette. This layer is discarded. The packed erythrocytes are washed twice in five volumes of 0.15 $M$ NaCl. After the last centrifugation the packed cells (volume = y ml) are hemolyzed by mixing with 9 y ml distilled water, and the stroma is centrifuged down at 10,000 $\times$ $g$ for 30 min. The supernatant hemolysate is adjusted to 40 y ml and 0.02 Molar in sodium phosphate buffer, pH 6.3. The hemolysate is then mixed with 0.8 y gm. (0.8 gm. dry weight per ml of initial packed erythrocytes) of diethyl amino ethyl cellulose (DEAE, Eastman Kodak Co.)[16] which has been equilibrated against 0.02 $M$ phosphate buffer, pH 6.3. The mixture is filtered on a Büchner funnel and rapidly washed with 0.02 $M$ phosphate buffer, pH 6.3, until the DEAE is free of hemoglobin. The enzyme is then eluted with 10 y ml of 0.5 $M$ phosphate buffer, pH 6.3. The eluate is brought to 70 per cent saturation (4°C) with solid ammonium sulfate. Mercaptoethanol is present in a 1:2,000 dilution in all solutions. The eluate is made 0.1 per cent (w/v) in ethylene diamine tetraacetic acid (EDTA), pH 7.0. The suspension of enzyme is concentrated to between $^1/_4$ y and y ml by centrifuging and discarding supernate. To each ml suspension is added 1.12 ml distilled water to bring the mixture to 33 per cent saturation with ammonium sulfate. After centrifugation, the supernate is isolated and brought to 53 per cent saturation by the addition of 0.42 ml of saturated ammonium sulfate per ml supernate. The precipitate is stored at 4°C, as a thick suspension, in the 53 per cent saturated ammonium sulfate. These steps result in a 60–80 fold purification of the G-6-PD and yield a preparation essentially free of hemoglobin and 6-PG dehydrogenase. Certain advantages in the use of this incompletely purified preparation have been cited.[4] That this preparation is by no means pure is indicated by column chromatography[4] and by the more recent achievement of over 10,000 fold purification of G-6-PD from normal blood.[17] Just prior to each experiment a small amount of suspension is dissolved in 0.5 ml distilled water, containing $10^{-5}$ $M$ TPN, and dialyzed for 6 hr against three changes of a solution which is 0.02 or 0.05 $M$ in phosphate buffer, pH 6.3, $10^{-5}$ $M$ in TPN, and 1:1,000 in mercaptoethanol. The ionic strength of

the dialysate is found to be essentially the same as unused dialyzing solution after this step. All purifications and storage are carried out at 4°C.

*Results.*—When a hemolysate was prepared by the addition of 9 or 19 volumes distilled water to the washed, packed erythrocytes from the infant with non-spherocytic hemolytic anemia, the activity of G-6-PD was observed to fall rapidly as the hemolysate stood at 0°C. The activity immediately after hemolysis was 1.6 O.D. units (at 340 mμ)/min, per ml packed cells, in an assay of 1 ml total volume and 1 cm light path (25°C). This corresponds to approximately 9 per cent of normal activity. The activity was <0.1 O.D. units, or essentially imperceptible, 3 hr after hemolysis. The rapid fall in activity was observed even after stroma was removed from the hemolysate. This lability was far greater than that which was observed under comparable conditions in hemolysates from normal or primaquine-sensitive humans.

Relatively small amounts of various substances stabilize or inactivate human G-6-PD; the stability also varies with the degree of dilution of the enzyme.[4, 11] In order better to distinguish whether the lability was due to extrinsic or intrinsic factors, G-6-PD from this individual with non-spherocytic hemolytic anemia was purified through the step of suspension in 53 per cent saturated ammonium sulfate.

TABLE 1

INACTIVATION, AT 49°C, OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM A CASE OF CONGENITAL NON-SPHEROCYTIC HEMOLYTIC ANEMIA

| | Activity* | |
|---|---|---|
| Time at 49°C | Tube A (H) | Tube B (H & N) |
| 0 min | $2.0 \times 10^{-3}$ | $3.9 \times 10^{-3}$ |
| 4 min | $0.3 \quad 10^{-3}$ | $2.0 \quad 10^{-3}$ |
| 7 min | $0.0 \quad 10^{-3}$ | $1.5 \quad 10^{-3}$ |
| 16 min | $0.0 \quad 10^{-3}$ | $1.3 \quad 10^{-3}$ |

* O.D. units/min per aliquot of 50 μL, assayed in 1.0 ml total volume; readings followed for 30–40 min. The assay mixtures were cleared of any turbidity by centrifugation prior to initiating the reaction with G-6-P.

The precipitate from this suspension was then diluted to approximately 2 mgm protein[18]/ml with 0.05 *M* phosphate buffer, pH 6.3, containing $10^{-5}$ *M* TPN. Two tenths ml was placed in each of two Pyrex centrifuge tubes (A and B). G-6-PD from a normal adult control was purified, as here described, to the step of suspension in 70 per cent ammonium sulfate. The suspension of G-6-PD (N) was then diluted tenfold in additional 70 per cent saturated ammonium sulfate and was centrifuged; 20 μl of supernate, containing no activity, was added to tube A, as a control. The G-6-PD (N) in this 70 per cent saturated ammonium sulfate was then resuspended and 20 μl, or approximately 3.5 γ protein, was added to tube B in order approximately to double the activity in B. Both tubes were incubated in a water bath at 49°C. The loss in activity on heating (Table 1) suggests, but by no means proves, that the erythrocytic G-6-PD (H) is intrinsically more labile than that from normal controls.

Low concentrations of TPN, which stabilize the G-6-PD from normal and primaquine-sensitive persons,[11] do not impart normal stability to G-6-PD (H) (Table 1). However, $10^{-5}$ *M* TPN is sufficiently stabilizing to permit storage of diluted G-6-PD (H) at 4°C for several days or to permit zero-order kinetics for several

hours at 25°C when assays of low activities of G-6-PD (H) are necessary. Adjustment of the hemolysates of H to $10^{-5}$ *M* in TPN in the purifications subsequently used resulted in a closer to normal percentage yield of G-6-PD.

To explore further the possible qualitative alterations in G-6-PD (H), the same catalytic parameters were studied as previously were examined[4, 12] in the G-6-PD from a normal, Caucasian, adult male (N) and from an asymptomatic negro adult male (S) with primaquine-sensitive erythrocytes, as manifested by an abnormal glutathione stability test[9] and by an activity of G-6-PD which was only 10 per cent of normal. Purification was carried through the dialysis step. Although the heat of activation was found to be essentially the same in the G-6-PD from all three sources (Fig. 1), striking differences were observed between H and either S or N in regard to the Michaelis constants (Figs. 2 and 3) and pH optimum (Fig. 4). In order to exclude the possibility that these differences might be due to new stocks of reagents,[19] age of the patient, or age of the erythrocytes, characterizations[20] were made on the partially purified G-6-PD from the erythrocytes of another normal (N′) and primaquine-sensitive adult (S′) and from an infant (N″) who had another type of hemolytic anemia associated with reticulocytosis comparable to (H). All values agreed with the previously determined catalytic parameters (Figs. 2–4) for G-6-PD from a normal adult.

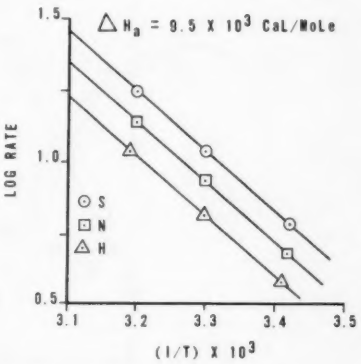


FIG. 1.—Arrhenius plot for heat or energy of activation for the G-6-PD from a congenital nonspherocytic hemolytic (H), primaquine-sensitive (S), and normal (N) individual. Each point represents the average of duplicate determinations. Temperature range 20–40°C.

The preparations of G-6-PD from N and S have been found to use the abnormal substrate 2-deoxy-glucose-6-phosphate at a maximal rate only 9 per cent of that for G-6-P. Their Michaelis constant for 2-deoxy-glucose-6-phosphate is 6.9 × $10^{-4}$ *M*.[12] Like the Michaelis constant for G-6-P, the constant for 2-deoxy-glucose-6-phosphate is some 4–5 times greater for H than for N. At concentrations of 6 × $10^{-4}$ molar the rate of utilization of 2-deoxy-glucose-6-phosphate compared to G-6-P was less with H than with N. This ratio more nearly approached the 9 per cent value when maximal velocities were estimated at infinite concentration of substrate.

DEAE column chromatography was performed on G-6-PD of N and H in the manner described elsewhere,[4] except that the concentration of sodium phosphate buffer was maintained at 0.02 *M* throughout, and the linear gradient was obtained with increasing concentrations of sodium chloride. Prior to chromatography the enzymes were purified and dialyzed as described here, the 33–53 per cent ammonium sulfate fractionation being omitted to provide lactic dehydrogenase as a marker. A mixture of equal activities of N and H moved off of the column as a single peak in a pattern like that for N alone. Aliquots from both an early and a late portion of the peak from the mixed preparation had activities at pH 9.75 which were 60 per cent of those at pH 7.5, indicating that these aliquots were still mixtures of

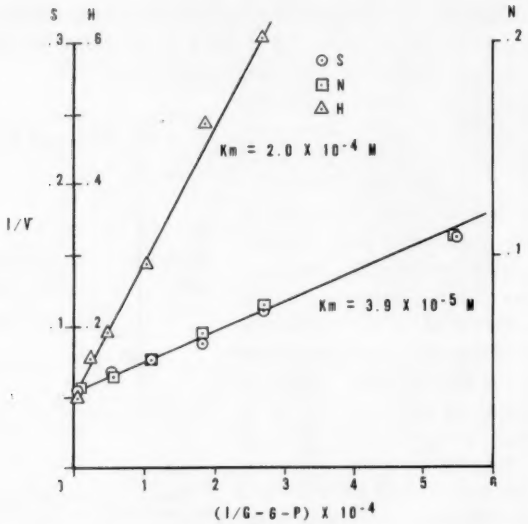


FIG. 2.—Lineweaver–Burk plot for Michaelis constant for G-6-P. Except for variation in G-6-P the assay conditions are as described.[12] Points for H represent the averages of duplicate determinations. For graphic comparisons the ordinates are scaled so as to superimpose the maximal velocities.

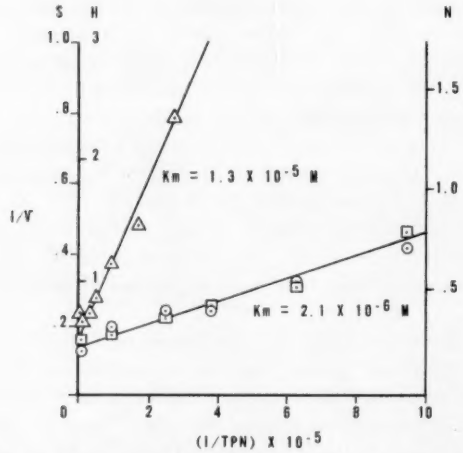


FIG. 3.—Lineweaver–Burk plot for Michaelis constant for TPN. Spectrophotofluorometer used to follow reaction. Except for variation in TPN the assay conditions are as described.[12] A comparable Km for TPN for H was obtained even with higher ($3 \times 10^{-3}$ *M*) concentrations of G-6-P. Points for H represent averages of duplicate determinations. Symbols as in Fig. 1.

equal activities of N and H (Fig. 4). The efficiency of the columns was indicated by wide resolution of the lactic dehydrogenase into three components, as has been observed by others.[21]

*Discussion.*—In the sense that the described purification steps and dialysis did not restore the catalytic characteristics of the G-6-PD of H to normal, this enzyme is qualitatively different from normal. The similar movement of the G-6-PD's from N and H on ion-exchange column chromatography suggests that the physical differences may be more subtle than the functional differences. The similarities of the heat of activation may be analogous to the failure to find a difference in heat of activation after crystalline aldolase has been functionally and molecularly altered by carboxy-peptidase.[22]

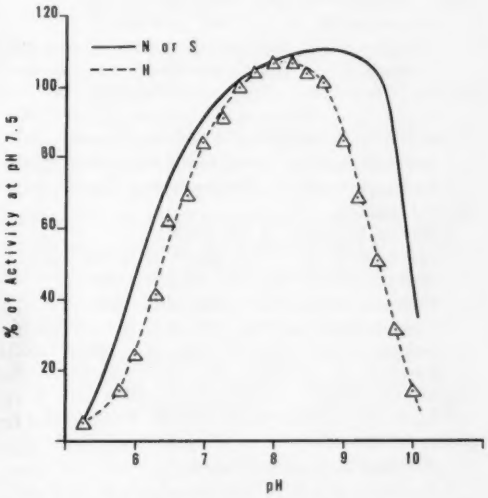


FIG. 4.—pH optima for G-6-PD's. Aliquots were removed at intervals of 0.25 pH units from an equimolar mixture of monosodium phosphate, tris(hydroxymethyl) aminomethane, and glycine, as the mixture was made more basic or acidic with NaOH or HCl. Final concentration, 0.05 *M* each in phosphate, Tris, and glycine, in each assay. $MgCl_2$ omitted. The highly coinciding points for average values for S and N are not drawn.

In view of the similarities between the G-6-PD of N and S, the recent report by Rimon *et al.*,[23] indicating an activator for human erythrocytic G-6-PD and a deficiency of this activation in S cells, is particularly interesting. The findings here reported indicate that yet another genetic and molecular mechanism, involving qualitative changes,[24] may operate to produce a deficiency in activity of human G-6-PD.

The anemia, or propensity for anemia, which seems to result from an impairment in erythrocytic G-6-PD, increases the likelihood that such persons will come under medical supervision and thereby facilitates selection of such mutants from the normal population. The existence of clinical variations[6, 9, 13, 14] in mutants involving G-6-PD suggests that even further genotypes and molecular expressions, warranting investigation, may exist.

*Summary.*—Details are given of the discovery of two different enzymic expressions by genotypes which have been previously known to effect a deficiency in human erythrocytic glucose-6-phosphate dehydrogenase. One expression results in a qualitative alteration of the enzyme, a phenomenon which hitherto has been difficult to ascertain in higher forms of life.

These studies were supported by grants from the National Institutes of Health, U.S. Public Health Service, and from the American Cancer Society. The authors wish to acknowledge the cooperation of Dr. Ernest Simon in procuring samples of S blood. Use of a spectrophotofluorometer was generously made available by Dr. Ranwel Caputto and his associates.

* The following abbreviations are used: G-6-P for α-D-glucose-6-phosphate; TPN for triphosphopyridine nucleotide; TPNH for reduced triphosphopyridine nucleotide; 6-PG for 6-phosphogluconolactone or 6-phosphogluconic acid.

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# *PRODUCTS OBTAINED BY DIGESTION OF THE NUCLEIC ACIDS OF SOME STRAINS OF TOBACCO MOSAIC VIRUS WITH PANCREATIC RIBONUCLEASE**

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Paper electrophoresis and paper chromatography were recently combined under conditions which resulted in a two-dimensional "mapping" procedure for the separation and determination of oligonucleotides.[1] This system provides the means to extend previous chemical studies[2–4] on mutant strains of tobacco mosaic virus (TMV). The proposed approach is to subject the ribonucleic acid (RNA) of each strain to the action of pancreatic ribonuclease (RNase), and then to separate and analyze the nucleotide and oligonucleotide fragments. Owing to the known specificity of action of RNase, the nature and quantity of the various products obtained by its action provide a clue to the sequential arrangement of nucleotides in the original polynucleotide chains. A similar approach has already been used with strains of TMV,[3, 4] but the higher resolution of the mapping procedure applied in the present instance allows the comparison of strains to be made in considerably greater detail. We are reporting here the results of such extensive analyses of the RNase digests of the nucleic acids from ordinary TMV and from the M and HR strains.

*Virus Strains.*—The common strain of TMV, and the M and HR strains were employed.[2, 5, 6] In the systemic host, Turkish tobacco, TMV evokes a green mottling of the leaves accompanied by some blistering and distortion; *M* produces no symptoms at all in this host and hence the name, masked; HR causes highly distinctive necrotic ring and thread-like patterns together with a very mild mosaic. On local lesion hosts such as *Nicotiana tabacum, var. xanthi*, TMV and M give very similar lesions whereas HR produces distinctly smaller spots. In derivation and other biological properties, M is considered closely related to TMV while HR is viewed as a distantly related strain.

Preparations of each strain were obtained by a process of differential centrifugation. Such preparations, when assayed in the electron microscope (through the courtesy of Dr. R. C. Williams), were found to consist of 75 per cent or more of the characteristic 15 × 300 mμ viral rods, the balance consisting primarily of rods of shorter length. The criteria for homogeneity of the strains were the same as described before.[7]

*RNA.*—RNA preparations were obtained from the virus by the phenol method of Gierer and Schramm,[8] modified slightly as previously described.[9]

*Digestion of RNA with Pancreatic Ribonuclease.*—Solutions containing about 8 mg. RNA, 0.3 mg. RNase (Worthington Biochemical Corporation, Freehold, N. J.), and 0.02 ml *M* sodium phosphate at pH 7.1 were held at 23° for 6–8 hr or at 37° for 24 hr. Aliquots of the digests were fractionated by the mapping procedure as described below.

*Determination of Amounts of RNA Mapped.*—To determine the amount of RNA analyzed, 25 microliters of each RNase digest were diluted with *N* KOH to 10

ml, held in a tightly stoppered volumetric flask for 24 hr at room temperature, and the optical density (D) at 260 mμ determined directly. This was done in duplicate, and the same 25 λ micropipet used throughout.

This procedure provided a uniform basis for comparing the strains, since they have essentially identical proportions of nucleotides.[2] To obtain the weight of RNA represented by the $D_{260\ m\mu}$ of the alkaline hydrolyzate, a common factor was used which was obtained as follows. The $D_{260\ m\mu}$ was calculated for an alkaline solution containing ribonucleotides in the proportions listed by Knight[2] using the extinction coefficients of Beaven *et al.*[10] for these nucleotides at pH 12. Corrections were then made on the basis of the experimentally determined change in $D_{260\ m\mu}$ when each of the nucleotides was taken from pH 12 to pH 14. Thus, the $D_{260m\mu}$ of 1 mg/ml of TMV-RNA hydrolyzate in *N* KOH was found to be 32.53. All optical measurements were made with a Beckman DU spectrophotometer. The weights of RNA obtained from spectrophotometric readings on the alkaline digests and use of the factor just given were found to agree within 6 per cent with the weights of RNA calculated from spectrophotometer readings at 258 mμ and use of the relationship $D_{258}$ of RNA at 1 mg/ml = 25.

*Mapping of Ribonuclease Digests of Viral RNA.*—At the completion of enzymatic action, 0.5 ml aliquots (equivalent to 2.5–3.5 mg of RNA) of the digest were slowly applied to Whatman 3 MM papers with an Ostwald-Folin pipet and "mapped" as previously described.[1] In the present experiments three electrophoresis boxes were used simultaneously, so that an RNA digest could be mapped in duplicate and a blank paper run in the third box. After the maps were obtained, contact prints were prepared from them according to the method of Smith and Allen.[11]

*Qualitative and Quantitative Determination of the Nucleotides and Oligonucleotides.*—Identification of the spots on the maps was by comparison of positions with the spots on a standard map on which the compounds had been identified.[1]

For quantitative analysis the mono- and oligonucleotide spots were located under ultraviolet light, marked, cut out, and eluted with 0.01 *N* HCl by shaking 3–6 hr. Equivalent areas from the blank map were eluted simultaneously. The pH of the eluates remained constant. The $D_{260}$ mμ values of all isolated fractions were then read against the proper paper blanks. The readings were converted into quantities of compound by use of published extinction values[10] either directly, as for U and C,[13] or to calculate extinctions for oligonucleotides based on their compositions. $D_{260}$ mμ values for 1 μgm of compound per ml at pH 2 are listed in Table 2. No allowance was made for the hypochromic state of the oligonucleotides.

The insoluble residue,[1] that is, the material still found in the electrophoresis band after the completion of the paper chromatography step, was eluted with *N* KOH for 24 hr at room temperature and its $D_{260}$ mμ value was read against that from a similarly treated blank. The amount of optical density thus recovered was compared with that applied to the paper, the latter value having been obtained from the alkaline hydrolyzate of an aliquot of the RNA digest (see above). The ratios of the constituent bases in the insoluble residue were determined by paper electrophoresis,[12] as described previously.[1]

*Results.*—When the RNA of each strain was digested with RNase under two sets of conditions, namely, 23° for 6–8 hr and 37° for 24 hr, it was found that essentially the same amounts of the compounds U, GU, and AU[13] were released.

From this it was concluded that at the enzyme-substrate ratios used the RNase digestion was complete after about 6 hr at 23°. Therefore, these conditions were employed in most instances during this investigation.

Table 1 gives an indication of the distribution with respect to solubility in the mapping solvents of the products eluted from maps of the RNase digests of TMV, M, and HR nucleic acids. In calculating the amounts of the soluble oligonucleotides isolated, the theoretical extinction coefficients were employed. Since this procedure does not allow for the hypochromic state of the oligonucleotides, their actual recovery is higher than the 61 per cent of total $D_{260}$ m$\mu$ indicated in Table 1.

TABLE 1

RECOVERY OF PRODUCTS FROM MAPPED RIBONUCLEASE DIGESTS OF THE RNA FROM STRAINS TMV*

| Fraction | TMV | M | HR |
|---|---|---|---|
| Soluble nucleotides† | 61.5 | 61.0 | 60.5 |
| Insoluble residue‡ | 26.4 | 25.7 | 27.3 |
| Total | 87.9 | 86.7 | 87.8 |

* Per cent of the total amount of optical density at 260 m$\mu$ applied to the paper.
† Soluble in the mapping solvents, see text. U and C and all included oligonucleotides are indicated in the key to Figure 1. The amounts of the oligonucleotides were obtained by use of calculated extinction coefficients, as described in the text, without allowance for hypochromicity. The actual recovery of the oligonucleotide fraction is thus better than indicated here.
‡ The experiments were done in triplicate and the results averaged. For a definition of the "insoluble residue" and for a description of the elution procedure see text.

About 25–30 per cent of the total $D_{260}$ m$\mu$ fractionated was recovered in the form of an "insoluble residue," the purine/pyrimidine ratio of which was found to be about 4.5/1. This ratio was found to increase with the length of time allowed for development of the maps in the paper chromatography step, and is apparently due to the gradual downward movement of smaller purine-rich oligonucleotides which trail in the solvent employed in this procedure.

In previous studies[1] with RNase digests of TMV-RNA, it was found that all of the discrete spots on the map represent single compounds, or mixtures of structural isomers, such as AGU-GAU. In the case of AGU-GAU and AGC-GAC, these groups of isomers were quantitatively resolved by an additional paper chromatography step with 30 per cent buffered ammonium sulfate. The other groups of structural isomers (Table 2, fractions 16–19) were not fractionated. The pentanucleotides $(A_3G)U$ and $(A_2G_2)U$ (Fig. 1) were not present in amounts sufficient for direct determination by elution from a single map.

Contact print maps of the RNase digestion products of each of the three strains are shown in Figures 1–3. It can be seen that the three maps are very similar in number and location of spots. It was concluded that the spots on maps of M and HR contained the same compounds as comparable spots on maps of TMV-RNA[1] since they exhibited the same ultraviolet absorption ratios for 250/260, 280/260, 290/260 m$\mu$ at pH 2, 7, and 12. The amounts of each nucleotide and polynucleotide were calculated from the $D_{260}$ m$\mu$ values as described in *Methods*. The average values thus obtained are presented in Table 2. It will be noted that any differences between the values for M and TMV are very small and probably within experimental error. However, several striking differences in values are apparent when the figures for HR are compared with those for TMV and M.

*Discussion.*—It is now commonly held that nucleic acid alone is the genetic

material of viruses. In this concept, the nucleic acid owes its specificity to the sequential arrangement of the nucleotides in the polynucleotide chain. Strains of TMV should constitute ideal material with which to verify the detailed chemical basis of such postulated genetic specificity, for there are many strains of TMV with characteristic biological and chemical properties, and it is relatively easy to get substantial quantities of highly purified preparations. However, there is at present no way to determine long polynucleotide sequences in a stepwise fashion, and the RNA of TMV seems to be a chain-like molecule of about 6,500 nucleotides. Fortunately, the great specificity of RNase provides a series of nucleotides and oligonucleotides whose nature and quantity in the digests must reflect to a considerable degree the nucleotide sequences in the original polynucleotide chain. Hence, if a reliable process is available for separating the oligonucleotide fragments

TABLE 2

SOLUBLE MONO- AND OLIGONUCLEOTIDES ISOLATED FROM MAPS OF RIBONUCLEASE DIGESTS OF RNA FROM TMV, HR, AND M*

| Fraction | Composition[19] | $D_{260}$ mμ, pH 2 for 1 μgm/ml | Micrograms compound/mg RNA mapped | | | Standard deviation† | | |
|---|---|---|---|---|---|---|---|---|
| | | | TMV | M | HR | TMV | M | HR |
| 1 | U | 0.0306 | 125 | 125 | 123 | 2.7 | 3.1 | 2.4 |
| 2 | C | 0.0210 | 81 | 80 | 78 | 2.0 | 2.7 | 2.8 |
| 3 | GU | 0.0324 | 56 | 55 | *62* | 1.4 | 1.9 | 1.6 |
| 4 | AU | 0.0369 | 53 | 51 | *47* | 1.5 | 1.4 | 1.7 |
| 5 | AC | 0.0318 | 47 | 46 | 43 | 1.1 | 1.2 | 1.1 |
| 6 | GC | 0.0274 | 42 | 41 | 41 | 1.1 | 1.0 | 1.3 |
| 7 | AAU | 0.0390 | 27 | 26 | *17* | 0.95 | 0.80 | 0.67 |
| 8 | AGU | 0.0360 | 21 | 21 | *15* | 1.6 | 1.6 | 1.7 |
| 9 | GAU | | 28 | 28 | 30 | 1.7 | 1.8 | 1.6 |
| 10 | GGU | 0.0330 | 14 | 13 | *20* | 1.0 | 0.96 | 1.4 |
| 11 | AGC | 0.0326 | 16 | 15 | 14 | 1.1 | 1.0 | 0.93 |
| 12 | GAC | | 17 | 17 | 16 | 0.95 | 0.94 | 0.90 |
| 13 | AAC | 0.0355 | 12 | 12 | *17* | 0.58 | 0.45 | 0.31 |
| 14 | GGC | 0.0297 | 9.8 | 9.9 | 9.4 | 0.72 | 0.68 | 0.83 |
| 15 | $A_3U$ | 0.0400 | 7.2 | 7.2 | 8.6 | 0.44 | 0.50 | 0.39 |
| 16 | $(A_2G)U$ | 0.0378 | 17 | 18 | *23* | 0.82 | 0.66 | 0.57 |
| 17 | $(AG_2)U$ | 0.0355 | 12 | 12 | *7.2* | 0.65 | 0.99 | 0.61 |
| 18 | $(AG_2)C$ | 0.0330 | 14 | 15 | *10* | 0.90 | 1.2 | 0.97 |
| 19 | $(A_2G)C$ | 0.0352 | 13 | 14 | 16 | 1.1 | 1.1 | 1.3 |
| 20 | $A_3C$ | 0.0376 | 4.0 | 4.0 | 9.0 | 0.14 | 0.18 | 0.25 |

* About 2.5–3.5 mg of at least three RNA preparations from each virus were mapped in duplicate, and the results averaged.

† Standard deviation of individual values from the mean. Eight analyses were made of TMV and HR nucleic acids and six of M. None of the values for M are considered significantly different from those listed for TMV. The most probably significant differences between HR and the other two strains are italicized in column 6.

after RNase action, much information on nucleic acid structure can be obtained even though a complete sequence is not determined. We feel that the mapping procedure as described here is such a process. The next step is to attempt to correlate such analytical data with other properties of the virus. This cannot yet be done in specific detail, but it can be done in a general way, as follows:

The specific action of TMV nucleic acid is presumably manifested in one or both of two ways: (1) in the chemical nature (that is, in amino acid sequences) of the protein with which the nucleic acid is normally combined, and (2) in the symptoms which the virus causes in susceptible hosts.

The protein components of TMV and M are so similar that no significant difference between them in composition or amino acid sequence has yet been observed.[7] In contrast, HR protein differs in numerous ways from TMV and M proteins,

although it shares common amino- and carboxyl-terminal features.[2] The three strains all differ from one another, as described above, with respect to symptoms which they cause in Turkish tobacco. Turning now to the nucleic acids, the results summarized in Table 2 indicate that there are at least ten quantitative

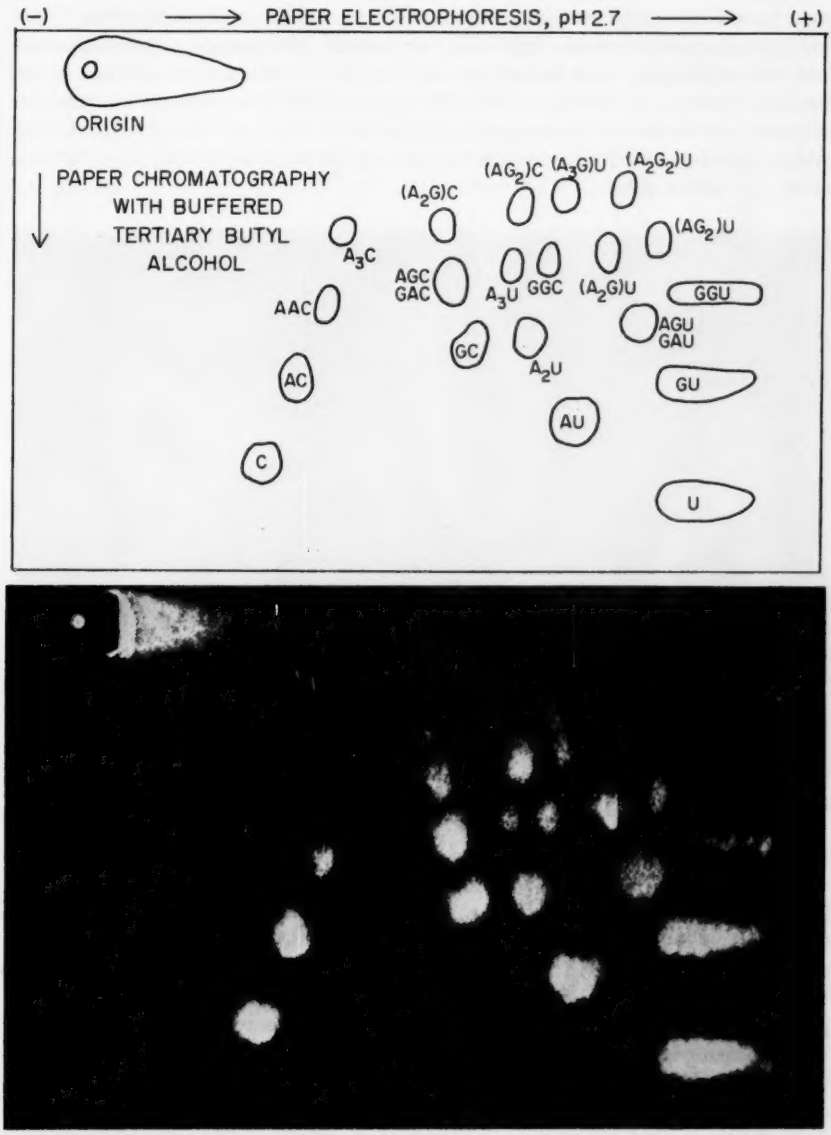


FIG. 1.—Contact print map, and key to map, of ribonuclease digestion products from 2.95 mg of TMV-RNA.

differences between TMV and HR nucleic acids in the frequencies with which certain oligonucleotide sequences appear. On the other hand, no positively significant differences of this sort are found between TMV and M nucleic acids. We do not interpret the latter as meaning that there are no differences between TMV and M in nucleotide sequence; the mapping procedure is neither complete enough nor accurate enough to warrant such a conclusion. However, it is clear that TMV and M nucleic acids must be very similar in structure, whereas it is equally apparent that HR nucleic acid must differ from the others in a number of sequences. These results in general are consistent with the postulate that the protein structure of a virus reflects the nucleotide sequences of its nucleic acid (or vice versa), but the comparison of M and TMV provides no basis yet for the different disease symptoms shown by these strains.

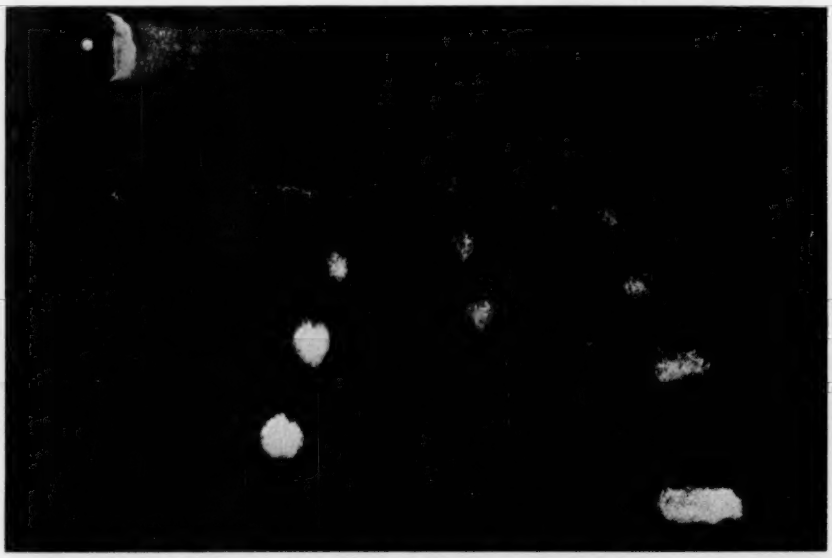

FIG. 2.—Contact print map of ribonuclease digestion products from 2.67 mg of M-RNA. See Fig. 1 for key.

The many similarities of the strain nucleic acids inferable from the data of Table 2 are perhaps as noteworthy as the differences. It is particularly interesting that the nucleic acids of all three strains yield essentially identical amounts of uridylic and cytidylic acids when treated with RNase. This must mean, on the basis of the known specificity of pancreatic RNase, that these strains contain clusters of pyrimidine nucleotides in essentially identical frequencies.

The present results confirm the three similarities and, qualitatively, two of the three differences between TMV and HR nucleic acids previously reported.[4] To this we can add about eight new differences between TMV and HR, as shown in Table 2, thus providing extensive chemical evidence for altered nucleotide sequences to match the distinct biological properties and protein structures of TMV and HR.

A similarity between TMV and M nucleic acids with respect to quantities of AU and GU released by RNase was recently noted,[15] and this was also observed by us; but none of the four previously reported differences between TMV and M nucleic acids was confirmed, including the claim that considerably larger amounts of C and U are released from M than from TMV.[16] As shown in Table 2, TMV and M were qualitatively and quantitatively indistinguishable in the twenty fractions examined. The only explanation we can offer at present for the divergencies between the earlier results and the present ones is that the mapping procedure is probably a more discriminating tool than the methods used before. In the mapping procedure, but not in the previously employed analyses of strain nucleic acids, it is possible to make comparative summations of the components in the various oligonucleotides differing in quantity and hence verify the analytical results. In other

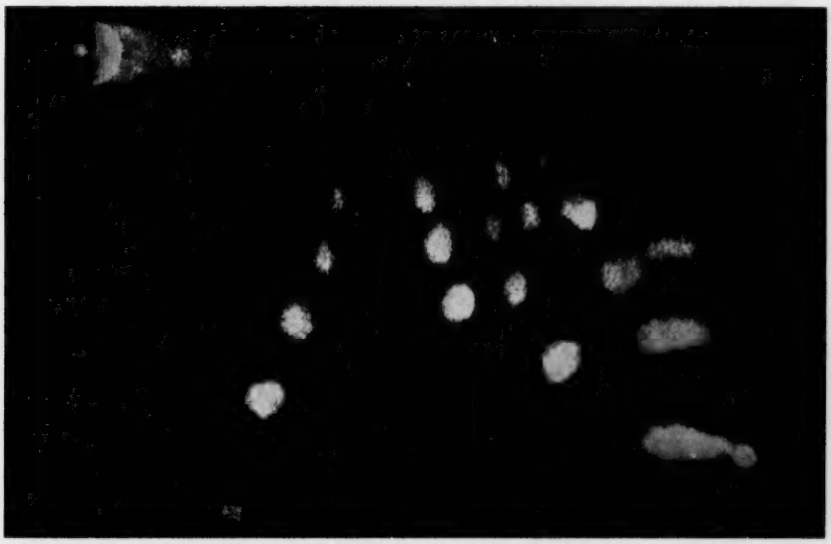

FIG. 3.—Contact print map of ribonuclease digestion products from 3.37 mg of HR-RNA. See Fig. 1 for key.

words, if TMV has more AAU than HR, HR must have more A and U in some other sequences to compensate for it. This is actually found to be the case when such summations are applied to the 10 points of difference between TMV and HR listed in Table 2. Another example of the efficiency of the mapping procedure is its demonstration of the presence of large amounts of the dinucleotide GC (see Figs. 1–3 and Table 2) which had been reported missing from RNase digests of TMV, M, and HR nucleic acids.[4, 15] Also, such RNase digests were previously claimed to have large amounts of AGC,[15] whereas the fraction labeled AGC by Reddi[15] is shown by the mapping procedure, and an additional chromatography step as described above, to be a composite of GC, AGC, and GAC.

*Summary.*—The nucleic acids of three strains of tobacco mosaic virus were digested with pancreatic ribonuclease and the resulting products were separated

into 20 fractions by a two-dimensional mapping procedure employing paper electrophoresis and paper chromatography. Maps of the three strains were qualitatively very similar. Quantitative analysis of the various fractions showed no significant differences between strains M and TMV, but revealed several striking differences between the strain HR and the other two strains. The differences brought out by the mapping procedure are considered to reflect altered patterns of nucleotide sequence in the original polynucleotide chains.

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## *ASSOCIATION CONSTANTS OF COMPLEX IONS: THE EVALUATION OF FREE LIGAND CONCENTRATION FROM MEASUREMENTS OF FREE METAL-ION CONCENTRATION**

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Although potentiometry is probably the most precise method for obtaining data from which association constants of complex ions may be calculated, difficulties are encountered when it is used to study certain systems. The purpose of this paper is to present experimental procedures which eliminate these difficulties. The procedures are restricted to systems in which only mononuclear species (i.e., species containing only a single metal-ion) are present and in which activity coefficients do not vary. A simple test is given for establishing that a system meets these re-

quirements. The primary result of the experimental procedures is the evaluation of $\bar{n}$, the average number of bound ligands per metal-ion, as a function of free ligand concentration. Secondarily, values of $\bar{n}$ at different free ligand concentrations may be used in calculating association constants by the methods discussed by Bjerrum and others.[1-3]

*Derivations.*—We shall begin by deriving some general equations which will be applied to experimental situations in the next section. For a system containing metal-ions, $M$, and ligand, $L$, which form mononuclear complexes, the definition of $\bar{n}$ gives

$$\bar{n} = \frac{(L)_0 - (L)}{(M)_0} = \frac{\sum_{i=1}^{n} i\beta_i(M)(L)^i}{(M) + \sum_{i=1}^{n}\beta_i(M)(L)^i} \tag{1}$$

where $(L)$ and $(M)$ are the concentrations of free ligand and free metal-ion, respectively; $(L)_0$ and $(M)_0$ are the total concentrations of these substances; $\beta_i$ is the concentration association constant for the species $ML_i$; and $n$ is the maximum number of ligands which can be bound per metal-ion. The equation is valid only when activity coefficients remain constant. Since $(M)$ cancels in the numerator and denominator on the right of equation (1), $\bar{n}$ is a function of $(L)$ only. Rearrangement of equation (1) gives

$$(L)_0 = (L) + \bar{n}(M)_0. \tag{2}$$

From equation (2) and the fact that $\bar{n}$ is a function of $(L)$ only, one obtains

$$\left(\frac{\partial(L)_0}{\partial(M)_0}\right)_{(L)} = \bar{n}. \tag{3}$$

We also note that, since $\bar{n}$ is a function of $(L)$ only, the partial derivative may be replaced by the ratio of finite differences at constant $(L)$. Hence

$$\bar{n} = \left(\frac{\Delta(L)_0}{\Delta(M)_0}\right)_{(L)}. \tag{4}$$

For a system containing two kinds of metal-ions, each of which forms mononuclear complexes with a ligand, one obtains for the total concentration of ligand

$$(L)_0 = (L) + \bar{n}(M)_0 + \bar{n}'(M')_0, \tag{5}$$

where the prime is used to distinguish the two kinds of metal-ions. The quantities $\bar{n}$ and $\bar{n}'$ may be defined by equations of type (1); each is therefore a function of $(L)$ only. Hence

$$\bar{n} = \left(\frac{\partial(L)_0}{\partial(M)_0}\right)_{(L),\,(M')_0} = \left(\frac{\Delta(L)_0}{\Delta(M)_0}\right)_{(L),\,(M')_0} \tag{6a}$$

$$\bar{n}' = \left(\frac{\partial(L)_0}{\partial(M')_0}\right)_{(L),\,(M)_0} = \left(\frac{\Delta(L)_0}{\Delta(M')_0}\right)_{(L),\,(M)_0}. \tag{6b}$$

*Applications.*—Any procedure for guaranteeing constancy of $(L)$ may be used in

conjunction with equations (4) or (6) to determine $\bar{n}$. We note that

$$(M)_0/(M) = 1 + \sum_{i=1}^{n} \beta_i (L)^i \tag{7}$$

is a function of $(L)$ only. Furthermore, since the $\beta_i$'s are positive, the function in $(L)$ is single-valued. Similarly, the ratio $(M)_0/(ML_i)$ is a single-valued function of $(L)$. Hence the provision that $(M)_0/(M)$ or $(M)_0/(ML_i)$ shall have the same value in two solutions establishes that $(L)$ is the same in the two solutions. Now, if the solution of metal-ion and ligand in contact with the metal corresponding to the metal-ion form a reversible half-cell, the potential of the half-cell relative to a standard half-cell† may be expressed by

$$E = a + \frac{RT}{\mathbf{NF}} \ln (M) = b + \frac{RT}{\mathbf{NF}} \ln \frac{(ML_i)}{(L)^i},$$

where $a$ and $b$ are constants if indifferent electrolyte is present in the working half-cell in sufficient quantity to prevent changes in activity coefficients as $(M)$ is varied and if experimental conditions have been arranged so that junction potentials are negligible. Under these conditions

$$E - \frac{RT}{\mathbf{NF}} \ln (M)_0 = a + \frac{RT}{\mathbf{NF}} \ln \frac{(M)}{(M)_0} = b + \frac{RT}{\mathbf{NF}} \ln \frac{(ML_i)}{(M)_0 (L)^i}$$

and hence $E - (RT/\mathbf{NF}) \ln (M)_0$ is a single-valued function of $(L)$.

To evaluate $\bar{n}$, one may proceed by using experimental E. M. F. data to plot two graphs of $E - (RT/\mathbf{NF}) \ln (M)_0$ *vs.* $(L)_0$. The systems represented by one of the graphs all have the same value of $(M)_0$, while the systems used for the other graph have a different, constant value of $(M)_0$. The two graphs intersect the ordinate in a common point, the value of which is equal to the cell potential at unit concentration of free metal-ion. The value of $\bar{n}$ is then calculated at each value of $E - (RT/\mathbf{NF}) \ln (M)_0$ by reading values of $(L)_0$ from the two graphs and applying equation (4). When $\bar{n}$ is known at known values of $(L)_0$ and $(M)_0$, equation (2) is used to calculate $(L)$. A preliminary check of the precision of the experimental data, of the absence of polynuclear species, and of the constancy of activity coefficients is obtained from a comparison of the values of $(L)$ calculated from equation (2) and the two sets of values for $(L)_0$ and $(M)_0$ obtained from the two graphs. A more sensitive test for these conditions is given later in this section.

This procedure was used on the data obtained by Leden[4] for cadmium chloride complexes. Unfortunately, the spacing of experimental points was such that the interpolation error is large. To within this error, however, the calculated $(Cl^-)$ agrees with the value measured by Leden with the aid of a Ag-AgCl electrode. For points with small interpolation error, the calculated chloride concentrations agree with the experimental values as well as do the values calculated by the method of successive approximations used by Leden.

If an electrically inert metal-ion, $M'$, is present in addition to the active metal-ion, the treatment is not markedly different. Now, however, three graphs of $E - (RT/\mathbf{NF}) \ln (M)_0$ *vs.* $(L)_0$ are required. The first graph represents systems containing known, constant values of $(M)_0$ and $(M')_0$. The systems represented by

the second graph have the same constant value of $(M)_0$ as those for the first graph but a different constant value of $(M')_0$. The third graph represents systems with $(M')_0$ equal to that for the systems of the first graph but with a different constant value of $(M)_0$. The three graphs intersect the ordinate in a common point, the value of which is equal to the cell potential at unit concentration of the free electrically active metal-ion. Figure 1 may clarify the procedure. Values of $(L)_0$ read from graphs (1) and (2) at a constant value of $E - (RT/\mathbf{NF}) \ln (M)_0$ and the two values of $(M')_0$ contained in the systems represented by these graphs are used to calculate $\bar{n}'$ from equation 6b. Values of $(L)_0$ from graphs (1) and (3) and the two values of $(M)_0$ are used to calculate $\bar{n}$ from equation (6a). Finally equation (5) may be used to evaluate $(L)$.

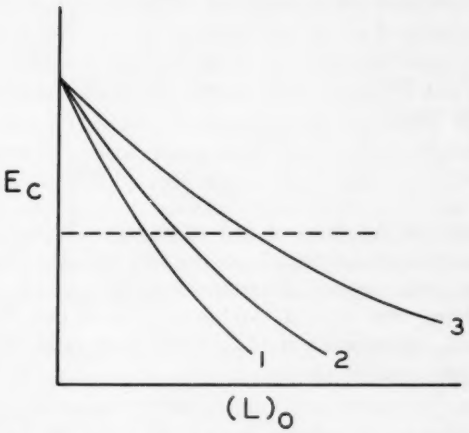


FIG. 1.—$E_c$, corrected emf, equals $E - (RT/\mathbf{NF}) \ln (M)_0$ if only one metal-ion is electrically active and equals $E - (RT/\mathbf{NF}) \ln [(M)_0/(M')_0]$ if both are active. Curve 1, $(M)_0 = C_1$, $(M')_0 = C_2$; Curve 2, $(M)_0 = C_1$, $(M')_0 = C_3$; Curve 3, $(M)_0 = C_4$, $(M')_0 = C_2$.

Fortunately, the data of Gross and Gryder[5] on copper and nickel tetrametaphosphate complexes permit the calculation of $\bar{n}_{Ni}$ by this procedure. The results are given in Table 1. The agreement between $\bar{n}$ calculated by this procedure and by the procedure used in the original paper is satisfactory when one considers the sensitivity of the older method to the values chosen for the copper equilibrium constants. It appears, however, that the limit of error placed on the equilibrium constants in the original work is too low.

We shall now consider a system in which the ions $M$ and $M'$ are the oxidant and conjugate reductant of an oxidation-reduction couple. If a reversible half-cell

TABLE 1

CALCULATION OF $\bar{n}_{Ni}$ FROM DATA OF GROSS AND GRYDER[5]
TOTAL $Cu^{++} = 2.575 \times 10^{-3} f$

| E.M.F. | Total* $P_4O_{12}^{-4}$ $\times 10^3 f$ | Total $Ni^{++}$ $\times 10^3 f$ | $\bar{n}_{Ni}$, this paper | Previous work |
|---|---|---|---|---|
| 10.44 | 2.225 | 0 | 0.258 | ... |
| 10.36† | 2.562 | 0 | 0.248 | ... |
| 10.36 | 10.20 | 30.84 | 0.248 | 0.253 |
| 10.13 | 5.11 | 10.28 | 0.278 | ... |
| 20.07 | 4.45 | 0 | 0.252 | 0.275 |
| 19.95 | 10.14 | 10.28 | ... | ... |
| 31.96 | 10.00 | 0 | 0.554 | 0.505 |
| 32.00 | 15.01 | 5.60 | ... | ... |
| 35.23 | 14.94 | 2.570 | 0.895 | 0.775 |
| 35.17 | 17.43 | 5.14 | 0.969 | 0.845 |
| | | | ... | 0.837 |

* Corrected for $NaP_3O_{12}^{-2}$, the correction being at most 4 per cent.
† Interpolated values.

is formed by the ions and an inert electrode, its potential relative to a standard half-cell is given by

$$E = a + \frac{RT}{\mathbf{NF}} \ln \frac{(M)}{(M')} = b + \frac{RT}{\mathbf{NF}} \ln \frac{(ML_i)(L)^{j-i}}{(M'L_j)} \tag{8}$$

where $a$ and $b$ are again constants if junction potentials and variation in activity coefficients can be neglected. For this system $E - (RT/\mathbf{NF}) \ln (M)_0$ is no longer a function of $(L)$ only and a new criterion for constancy of $(L)$ is required. Equation (7) and the analogous equation for $(M)_0/(ML_i)$ show that the ratios $(M)_0/(M)$, $(M')_0/(M')$, $(M)_0/(ML_i)$, and $(M')_0/(M'L_j)$ are all functions of $(L)$ only. Hence the ratios

$$\frac{(M)_0(M)}{(M')_0(M)} \quad \text{and} \quad \frac{(M)_0(M'L_j)}{(M')_0(ML_i)}$$

are also functions of $(L)$ only. We also note that these functions are single-valued because the $\beta_i$'s are positive. Hence the requirement that one of the latter ratios is constant guarantees constancy of $(L)$ except under the unlikely circumstance that $\beta_i = \beta_i'$ for all $i$, in which case the ratios are numerically equal to one, independent of $(L)$. Since these ratios are functions of $(L)$ only, it follows from equation (8) that

$$E - \frac{RT}{\mathbf{NF}} \ln \frac{(M)_0}{(M')_0}$$

is a function of $(L)$ only. Values of $\bar{n}$ and $\bar{n}'$ can therefore be calculated by a procedure identical to that for the last case with the one exception that

$$E - \frac{RT}{\mathbf{NF}} \ln \frac{(M)_0}{(M')_0}$$

is used instead of

$$E - \frac{RT}{\mathbf{NF}} \ln (M)_0.$$

Graphs (1) and (2) of Figure 1 are again used with equation (6b) to calculate values of $\bar{n}'$ and graphs (1) and (3) with equation (6a) to calculate $\bar{n}$. For this case the three graphs intersect the ordinate at a common point, the value of which corresponds to the cell potential at unit ratio of concentrations of free oxidant and reductant. The slope of the curves will be positive or negative depending upon whether the reductant or oxidant forms the stronger complex. The author's search of the literature failed to reveal a set of experiments so designed that this treatment could be tested.

A simple test for the applicability of the above procedures to a given experimental system is easily made. Since, for mononuclear species, $\bar{n}$ is independent of $(M)_0$, the same results for $\bar{n}$ as a function of $(L)$ will be obtained at widely different values of $(L)_0$, $(M)_0$, and $(M')_0$. If on the other hand polynuclear species are formed in reasonable quantity, $(M)_0/(M)$ will be a function of $(M)$ as well as $(L)$. If sizable changes in activity coefficients occur, the apparent association constants will vary

and $(M)_0/(M)$ will be a function of $(M)_0$, $(M')_0$, and $(L)_0$ as well as of $(L)$. In either case, the apparent value of $\bar{n}$ obtained at widely different values of $(M)_0$, $(M')_0$, and $(L)_0$ but at apparently constant values of $(L)$ will be different. If such variation in $\bar{n}$ is obtained, it may be concluded that polynuclear species exist or that large variations in activity coefficients occur. This behavior of $\bar{n}$ is observed for Leden's data on complexes of cadmium bromide.[4] Leden found that he could fit his data only by assuming polynuclear species.

Although the discussion has been limited to data obtained by potentiometry, it is evident that the method is more general. Any experimental procedure which can be used to measure a quantity proportional to $(M)f(L)$, where $f(L)$ is a single-valued function of $(L)$, can be used to establish constancy of $(L)$ since $(M)f(L)/(M)_0$ is then a single-valued function of $(L)$. This has been recognized previously for spectrophotometric data.[6] If only mononuclear species occur in each of two places, the quantity of metal-ion extracted divided by total metal-ion remaining in the aqueous phase is a single-valued function of $(L)$ and may be employed directly. A similar result applies for cation exchange methods. For data obtained by the last two procedures, one must correct $(L)_0$ in the aqueous phase for any loss to the second phase and should expect larger activity effects because of changes occurring in the nonaqueous phase.

*Summary.*—A direct experimental procedure which does not involve successive approximations or graphical differentiation is given for determining the average number of bound ligands per metal-ion as a function of the concentration of uncomplexed ligand in systems containing one or more kinds of complexing metal-ions from measurements of the free concentration of one of the metal-ions or the ratio of the free concentrations of two of them. Potentiometric procedures are therefore directly applicable to systems which contain an oxidation-reduction couple, both members of which form one or more complexes with a ligand. The method is restricted to systems which form only mononuclear complexes and in which activity coefficients remain constant. A simple test for these conditions is given.

The author is grateful to Professor W. Mansfield Clark for helpful discussion.

* This work was supported in part by grant G2745 from the National Science Foundation.

† For the purposes considered here, any reference half-cell is satisfactory. However, as an aid to workers with other interests, the particular reference half-cell should be standardized.

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# *DESIGN AND SYNTHESIS OF A CATALYST FOR THE AEROBIC OXIDATION OF CYTOCHROME C*,†*

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The oxidation of cytochrome *c* by molecular oxygen is known to be specifically catalyzed by cytochrome oxidase or cytochrome *a*. At pH = 7 the mid-point reduction potentials of cytochromes *a* and *c* were reported to be 0.29 and 0.26 *v*, respectively.[1] Since only one electron is removed from each ferrocytochrome *c* molecule when the latter is oxidized to ferricytochrome *c*, it is of interest to investigate the possibility that the aerobic oxidation of cytochrome *a* also takes place through a one-electron transfer mechanism.

Assuming that the aerobic oxidation of cytochrome *a* takes place through a one-electron transfer mechanism, the first reduction product of molecular oxygen must be $O_2^-$ or $HO_2$ which presumably can rapidly further oxidize other cytochrome *a* molecules. A summary representation of such a reaction path in a buffered solution would be as follows.

Step I:

$$O_2 + a \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} O_2^- + a^+;$$

$$O_2^- + H_2O \rightleftharpoons HO_2 + OH^-;$$

Step II:

$$HO_2 + a \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} HO_2^- + a^+;$$

$$HO_2^- + H_2O \rightleftharpoons H_2O_2 + OH^-;$$

Step III:

$$H_2O_2 + a \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} HO + OH^- + a^+;$$

Step IV:

$$HO + a \xrightarrow{k_4} OH^- + a^+,$$

where *a* and $a^+$ represent ferro- and ferricytochrome *a*, respectively, and $k_1$, $k_2$, etc., are the rate constants.

At the half-period of this reaction, when $t = t_{1/2}$,

$$[a] = [a^+] \equiv C,$$

let us define

$$R \equiv -\left(\frac{d[a]}{dt}\right)_{t_{1/2}} = \left(\frac{d[a^+]}{dt}\right)_{t_{1/2}},$$

$$R_I \equiv C(k_1[O_2] - k_{-1}[O_2^-]),$$

$$R_{II} \equiv C(k_2[HO_2] - k_{-2}[HO_2^-], \text{ etc.}$$

we have

$$R = R_{\mathrm{I}} + R_{\mathrm{II}} + R_{\mathrm{III}} + R_{\mathrm{IV}}.$$

The steady-state condition requires that

$$R_{\mathrm{I}} = R_{\mathrm{II}} = R_{\mathrm{III}} = R_{\mathrm{IV}},$$

and in general we have

$$R_{\mathrm{I}} \geqq R_{\mathrm{II}} \geqq R_{\mathrm{III}} \geqq R_{\mathrm{IV}}.$$

$\therefore$

$$\frac{R}{C} \eqq \frac{4R_{\mathrm{I}}}{C} < 4k_1[\mathrm{O}_2]. \tag{1}$$

Using $-0.8v$[2] and $+0.3v$ as the approximate mid-point reduction potentials of the couples $O_2/O_2^-$ and $a^+/a$, respectively, at pH = 7 and 25°C, we have

$$\frac{k_1}{k_{-1}} = e^{-\Delta F^\circ/(RT)} = e^{(-0.8-0.3)(23.06)/(0.6)} = 4 \times 10^{-19}. \tag{2}$$

But $k_{-1}$ cannot be greater than the diffusion-limited bimolecular rate constant $k$ which we may estimate by assuming that the relative diffusion coefficient for the reacting species $D_{12} = D_1 + D_2 \eqq 2.5 \times 10^{-5}$ cm²/sec and that the "collision diameter" at the reaction site $\eqq 4 \times 10^{-8}$ cm as follows.[3]

$$k \eqq 4\pi(4 \times 10^{-8})(2.5 \times 10^{-5}) \left(\frac{6.02 \times 10^{23}}{1{,}000}\right) f = 7.5 \times 10^9 f$$

where $0 < f \eqq 1$ is a geometric factor. Thus $k \eqq 7.5 \times 10^9\ M^{-1}\ \mathrm{sec}^{-1}$, and hence from (2),

$$k_1 = 4 \times 10^{-19}\, k_{-1} \eqq 4 \times 10^{-19}\, k \eqq 3 \times 10^{-9}\ M^{-1}\ \mathrm{sec}^{-1}. \tag{3}$$

Since $[O_2]$ in air-saturated water at room temperatures is about $3 \times 10^{-4}\ M$, we get from (1) and (3)

$$\frac{R}{C} < 4 \times 10^{-12}\ \mathrm{sec}^{-1}.$$

Consideration of the electrostatic interaction[4] between $O_2^-$ and $a^+$ could raise the estimated limiting value by a factor of about 10. This would lead to

$$\frac{R}{C} < 4 \times 10^{-11}\ \mathrm{sec}^{-1}.$$

There is considerable uncertainty in the value of the mid-point potential chosen above for the couple $O_2/O_2^-$. But even if we choose the highest value of $-0.53v$ estimated by Latimer,[5] we get

$$\frac{R}{C} < 10^{-6}\ \mathrm{sec}^{-1},$$

which still corresponds to a half-life of about a week for this reaction. Since such a

rate would make aerobic life incredibly slow, we conclude that the aerobic oxidation of cytochrome *a* does not take place through such one-electron transfer steps.

One may still attempt to defend the one-electron transfer mechanism by assuming that Step I above is made thermodynamically more favorable through the participation of an unidentified metal ion or functional group, $X$, of the cytochrome *a* molecule as follows:

$$O_2 + a + X \rightleftharpoons a^+ + XO_2^-.$$

But in view of the arbitrary nature of assigning oxidation numbers, this is operationally identical to a two-electron transfer mechanism whereby the $O_2$-molecule is reduced to the peroxide state by capturing an electron from *a* and another one from $X$ in one step.

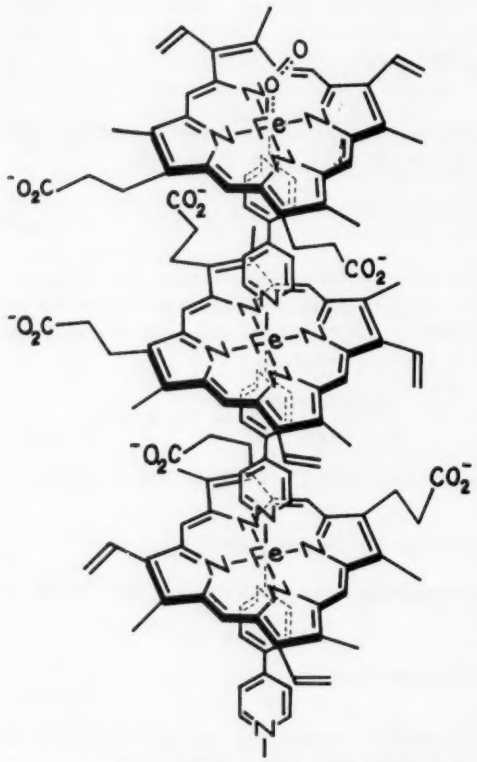


FIG. 1.—Structure of the active center of a synthetic catalyst for the aerobic oxidation of cytochrome *c*.

While the molecular mechanism of cytochrome oxidase action is still unknown, the above discussion suggests the possibility of designing, with the help of our present knowledge on the nature of the chemical bond, a synthetic molecule which catalyzes the aerobic oxidation of cytochrome *c* through a two- or four-electron transfer mechanism. In order to function efficiently, the catalyst must satisfy the following requirements: (*a*) It has a mid-point reduction potential equal to or higher than that of cytochrome *c;* (*b*) the reduced catalyst combines rapidly with molecular oxygen; (*c*) it is capable of reducing molecular oxygen through a two- or four-electron transfer mechanism; (*d*) the oxidized catalyst can rapidly capture electrons from ferrocytochrome *c* molecules.

These properties are incorporated in a type of polymer prepared in the present work by reacting heme, a bidentate ligand and a polycation in the proper order described later in the experimental part of this article. The structure of the active group of one of these polymers is illustrated in Figure 1. In contrast to ordinary bright red hemochromogens, the polymer in Figure 1 is purplish black in its Fe(II)-state and is insoluble in water. In its Fe(III)-state the binary copolymer with the simple structure illustrated in Figure 1 is unstable and tends to disintegrate or even

dissolve in alkaline aqueous medium. In the present work the simple binary polymer in Figure 1 is combined with half the equivalent amount of poly-L-lysine which presumably forms salt linkages with the negatively charged heme units and hence retards the disintegration of the polymer when the latter is oxidized on exposure to air. Since the replacement of water by 4,4′-dipyridyl molecules at the fifth and sixth coordinating positions of heme stabilizes the Fe(II)-state more than the Fe(III)-state, the polymer in Figure 1 is expected to have a mid-point reduction potential higher than that of hematin/heme couple. Equilibrium measurements show that the freshly prepared polymer has a mid-point reduction potential of *ca.* $0.3v$ which is indeed much higher than the value $-0.2v$ for the hematin/heme couple in aqueous solution at pH = 7. Moreover, since the different heme units in this

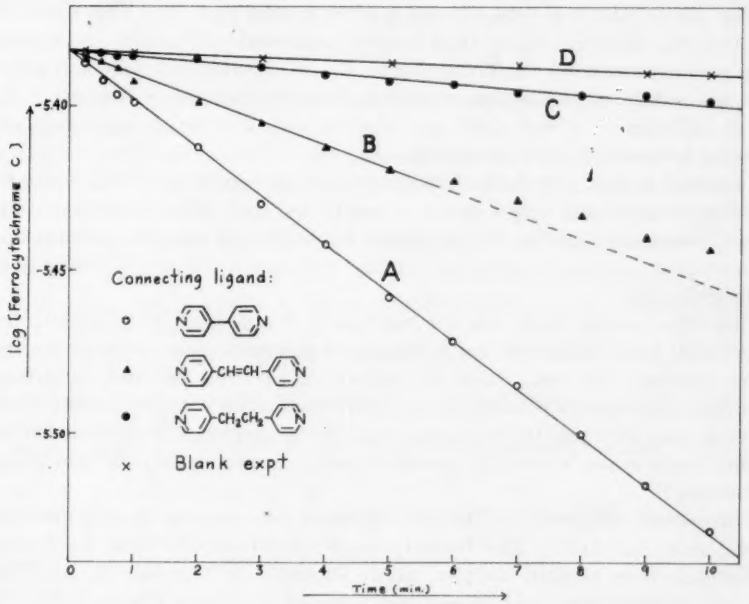


FIG. 2.—Catalytic oxidation of cytochrome *c* by molecular oxygen.

polymer are linked to each other through a conjugated double-bond system, the energy barrier for electron transfer between the different heme units should be relatively low. Therefore, we would expect each oxygen molecule bound to the terminal heme units in the Fe(II)-polymer to be reduced to water through a four-electron transfer mechanism. The Fe(III)-polymer may then be reduced by cytochrome *c* and reoxidized by molecular oxygen, etc.

This solid polymer was indeed found to catalyze the oxidation of cytochrome *c* by air with remarkable efficiency. A typical set of kinetic measurements is illustrated in Figure 2. Curve *A* in Figure 2 represents an experiment with 3.3 $\mu$g-equivalent of the catalyst per liter made with 4,4′-dipyridyl as the connecting ligand. Curves *B* and *C* represent similar experiments using catalysts made

with 1,2-di-(4-pyridyl)ethylene and 1,2-di-(4-pyridyl)ethane, respectively, as the connecting ligand. Curve *D* represents a blank experiment without catalyst. It is apparent from curve *A* in Figure 2 that the polymer made by combining heme, 4,4′-dipyridyl and poly-L-lysine is remarkably efficient in catalyzing the oxidation of ferrocytochrome *c* by air. The half-life of ferrocytochrome *c* in air-saturated aqueous solution was found to be inversely proportional to the amount of catalyst added. With 3.3 $\mu g$-equivalent of catalyst per liter, the half-life computed from the measured initial rate is 37 min at room temperature and pH 7.7. The observed rate starts to deviate noticeably from the first-order law after the reaction has taken place for more than an hour, due essentially to the gradual disintegration of the catalyst. In spite of this, the observed ratio of the number of moles of ferrocytochrome *c* oxidized after 90 min of reaction to the total number of equivalents of catalyst used is often greater than 10. This shows that we have true catalysis rather than coupled oxidation. Similarly, the insoluble black polymer made by combining heme, 1,2-di-(4-pyridyl)ethylene and poly-L-lysine also catalyzes the aerobic oxidation of ferrocytochrome *c* (see curve *B* in Fig. 2), although it is less stable and disintegrates at a much faster rate when subjected to repeated oxidation-reductions.

In contrast to these, the insoluble polymer made by combining 1,2-di-(4-pyridyl)-ethane with heme and poly-L-lysine is bright red and possesses little catalytic activity (see curve *C* in Fig. 2). Similarly, the bright red insoluble polymer made by combining 1,4-diaza-bicyclo[2,2,2]octane with heme and poly-L-lysine is also catalytically inert.

These observations show that the conjugated double-bond system which links the different heme units together in the active polymers plays an important part in the observed catalysis. Since the transfer of electrons between the different heme units is greatly facilitated by the connecting ligands with conjugated double-bonds, we may infer that in the present reaction the oxygen molecule bound at each terminal heme group is reduced directly to water through a four-electron transfer mechanism.

*Experimental.—Materials:* The 4,4′-dipyridyl was prepared by the method of Smith,[6] m.p. 112–113°. The trans-1,2-di-(4-pyridyl)ethylene and 1,2-di-(4-pyridyl)ethane were purified samples kindly supplied by Professor R. M. Fuoss. The cytochrome *c* was from horse-heart supplied by Sigma Chemical Co. The poly-L-lysine was obtained in the form of hydrobromide from Mann Research Laboratories, with reported average molecular weight of 175,000 and intrinsic viscosity of 0.55 in 0.2 F NaCl solution.

*Preparation of catalysts:* 2 mg (3.1 $\mu$mole) of hemin and 15.4 $\mu$equivalent of the appropriate connecting ligand were dissolved in 5 ml of warm 0.05 F KOH solution. The mixture was reduced with an excess of sodium dithionite solution, and then mixed with 3 ml of 0.2 F tris-hydroxymethylaminomethane buffer (pH 7.65) containing 0.65 mg (3.1 $\mu$mole) of poly-L-lysine. After standing for 10 min the black ppt. was centrifuged down, washed twice with 7–8 ml of Tris buffer containing 1 mmole/l of the connecting ligand. The solid polymer was then resuspended in the same buffer solution and triturated with a tissue grinder. Aliquots of this suspension were used immediately.

*Preparation of cytochrome c solution:* An aqueous solution containing 2 mg. of

cytochrome *c* per ml was reduced with hydrogen gas in the presence of a small amount of palladium black until the cytochrome *c* appears to be 80–90 per cent reduced. The excess hydrogen was removed by sweeping the solution with nitrogen. The solution was filtered, allowed to stand for several hours in air at room temperature, and stored at 0°C as stock solution.

*Rate measurements:* The reaction was followed by observing the change in optical density at 550 mμ by means of a Cary Recording Spectrophotometer. All measurements were made in 0.1 F Tris buffer (pH 7.7) containing 0.0005 mole of the connecting ligand per liter of the solution at 22 ± 3°C. The total cytochrome *c* concentration was determined by reducing the solution with a slight excess of $Na_2S_2O_4$ and calculating from the optical densities (O.D.) of the reduced solution by means of the expression

$$[\text{ferrocytochrome } c] = \frac{(\text{O.D.})_{550\ m\mu} - (\text{O.D.})_{530\ m\mu}}{2.0 \times 10^4}.$$

The cytochrome *c* solutions are approximately 70 per cent reduced at the beginning of the rate measurements.

A blank experiment with cytochrome *c* dissolved in buffer solution containing 0.0005 mole/liter of the connecting ligand but without added catalyst was made concurrently with each experiment. These blank rates were subtracted from the observed rates of catalyzed reactions before the computation of the rate constants.

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## *TISSUE CULTURE POPULATIONS AND THEIR RELATION TO THE TISSUE OF ORIGIN**

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*Introduction.*—Cultures of continuously propagated mammalian cells are usually devoid of properties specific to the tissue of origin. In fact, cultures derived from different tissues bear a remarkable similarity to one another. Among the many properties common to tissue cultures derived from a single species, irrespective of their tissue of origin, or whether malignant or normal, are antigenic specifi-

city,[1, 2] a requirement for certain amino acids nonessential for whole animal nutrition,[3] enzyme levels,[4] pattern of carbohydrate metabolism,[5] and sensitivity to chemotherapeutic agents.[6, 7] The various explanations proposed for this phenomenon can be summarized and simplified in the form of alternative hypotheses. According to the first hypothesis, tissue culture populations are descendants of the parenchymal cells of the tissue of origin, but in the process of multiplication these cells lose their differentiated properties and gain new properties in response to the dictates of the *in vitro* environment. In compliance with current usage, this hypothesis will be referred to as the dedifferentiation hypothesis. According to the second hypothesis, tissue culture populations are not derived from the parenchymal cells of the tissue of origin, but are descended from a ubiquitous minority cell type of the inoculum. The latter will be referred to as the selection hypothesis. In an attempt to distinguish between these two possibilities the following experimenta plan was adopted. Cultures were initiated from liver of day-old rats. After a brief period of growth, the nonviable portion of the inoculum was separated from the growing population. The attached and stretched cells (i.e., the growing cells) were examined for liver specific properties. According to the dedifferentiation hypothesis, one would expect a gradual and selective loss of liver specific properties rather than a simultaneous and complete loss of all liver properties. According to the second hypothesis, since the growing population is descended from cells not possessing liver specific properties, there should be a complete absence of liver specific properties at all times. Finally, liver inocula were pretreated with antisera specific against liver and antisera specific against tissue cultures and cultured in the absence of antisera. According to the dedifferentiation hypothesis, pretreatment with antiliver antiserum should destroy those cells which generate the ultimate tissue culture population and, as a consequence, completely inhibit subsequent growth. On the other hand, the selection hypothesis predicts that pretreatment of liver inocula with antitissue culture antiserum should, while leaving the liver parenchyma intact, destroy those cells destined to give rise to the final population. The markers used in these studies were liver antigenicity, ornithine transcarbamylase activity, and serum albumin content.

*Experimental Methods and Materials.—Media:* Eagle's Hela Medium,[8] with the addition of serine at 0.2 mg/ml and inositol at 0.4 mg/ml, was employed as the standard synthetic medium. The culture medium consisted of synthetic medium plus 15 per cent horse serum and 5 per cent fetal calf serum. All cultures were grown in 60 × 15 mm Petri dishes and incubated at 37°C in an atmosphere of 5 per cent $CO_2$ and 95 per cent air.

*Inocula:* One-day-old Sprague Dawley rats were sacrificed by decapitation and blood removed by perfusion of the whole body with saline injected into the left ventricle. The liver was then removed and sliced with a McIlwain tissue microtome in two directions at a setting of 0.2 mm. The resulting inocula had a distribution of sizes ranging from single cells to pieces of tissue 1.0 mm in diameter. When a suspension of single cells was required, the setting on the microtome was reduced to 0.05 mm, the liver sliced and suspended in neutralized synthetic medium, and the suspension forced through a 25 gauge needle with a syringe. To remove cellular debris both types of inocula were washed in the synthetic medium neutralized to pH 7.0. Single cell suspensions of adult liver were produced in a similar

manner except that the animals were perfused by injection of saline through the hepatic portal vein and drainage through the severed inferior vena cava.

Ornithine transcarbamylase assays were performed according to the method of M. E. Jones *et al.*[9] Serum albumin was assayed by a sensitive micro complement fixation technique developed by E. Wasserman and L. Levine.[10] Protein was determined by the method of Lowry *et al.*[11] with crystalline bovine serum albumin as standard.

*Antisera:* Rabbit antisera were prepared by the following immunization schedule. Each antigen at a level of $5 \times 10^6$ cells/ml was thoroughly washed in saline, suspended in Freund's adjuvant, and injected in two subcutaneous and two intramuscular depots. This was repeated after a three-week interval. One month later the rabbits received an intravenous injection of $10^6$ cells in saline, and were bled after seven to ten days. Subsequently, the animals received intravenous booster injections at monthly intervals and serum was taken after a week. To obtain anti-rat-liver antisera, purified single cell suspensions of adult rat liver were used. For production of antisera to tissue cultures, cells were harvested by trypsinization of tissue cultures, and the trypsin removed by washing.

Absorption of antisera was performed either with suspensions of adult rat liver cells or with a minced preparation of hepatectomized day-old rats. Both preparations were thoroughly washed with saline prior to use. For each 10 ml of antisera, 1.0 gm. of tissue was added, and the resulting suspension incubated with frequent agitation for 30 min at room temperature. The absorption material was then removed by centrifugation. This procedure was repeated three times.

*Experimental.*—1. *Antigenic properties of cultures derived from day-old rat liver:* One of the properties commonly used to describe and characterize a particular cell type is its antigenic structure. It was of interest, therefore, to examine the antigenic relationship between cells that become established in tissue culture, and the tissue of origin of these cells. Accordingly, an inoculum prepared from day-old rat liver was permitted to grow for twelve days. The plates were carefully washed to remove all cells that were not fixed to glass. The attached and stretched cells were freed by treatment with trypsin, harvested, and replated in culture medium in the presence of various rabbit sera. Each plate was inoculated with $10^4$ cells, and the rabbit sera added at a level of 3 per cent (v/v). After further incubation for $2^1/_2$ days, the plates were washed, the remaining cells fixed with 10 per cent neutral formalin and stained with Giemsa, and the attached and stretched cells counted. The rabbit sera employed were (1) pre-immunization serum designated as normal serum, (2) antiserum prepared against purified single cell suspensions of adult rat liver, (3) antiserum prepared against tissue cultures obtained from day-old rat kidney. Separate portions of the three sera were absorbed with either adult rat liver or minced preparations of hepatectomized day-old rats. The results of the experiment are recorded in Table 1. From the data it is apparent that both antisera, when unabsorbed, effectively destroy the ability of tissue culture cells from day-old rat liver to attach and stretch. Of importance, however, was the finding that liver culture cells were susceptible to anti-kidney-culture antiserum which had been absorbed with rat liver, while, at the same time, resistant to the action of anti-liver antiserum absorbed with hepatectomized day-old rat. These results implied that tissue culture populations derived from day-old rat liver dif-

TABLE 1

VIABILITY OF LIVER CULTURE CELLS IN THE PRESENCE OF RABBIT SERUM

| Serum | Unabsorbed | Absorbed with Adult Liver | Absorbed with Hepatectomized Day-Old Rat |
|---|---|---|---|
| | Number of attached and stretched cells per plate | | |
| Normal | 1005 | 2020 | 1750 |
| Anti-adult liver | 22 | 1260 | 1425 |
| Anti-day-old-rat-kidney culture | 9 | 55 | 1300 |

Liver culture cells were harvested by trypsinization and plated in the presence of various rabbit sera. The inoculum was $10^4$ cells per plate and the antiserum level was 3 per cent (v/v). After 60 hr incubation, the plates were washed, fixed with formalin, stained with Giemsa, and the number of attached and stretched cells counted.

fered from liver parenchymal cells in that they lacked certain liver antigens. Moreover, these same populations possessed antigens related to kidney culture antigens, which are absent in liver parenchyma. Of additional interest was the fact that absorption of all antisera with hepatectomized day-old rat eliminated toxicity. This implied that the antigens responsible for antiserum sensitivity of the liver cultures must, therefore, be present in tissues other than liver.

2. *Demonstration of anti-liver antibodies by complement (C′) fixation:* Central to the inference that culture cells differ markedly in antigenic structure from the original parenchymal cells is the assumption that anti-liver antiserum, absorbed with hepatectomized young rat does contain antibodies to the original parenchymal cells in spite of being ineffective against culture cells. This was confirmed by the use of complement fixation. The absorbed antiserum, in dilutions ranging from $^1/_{25}$ to $^1/_{800}$, was set up in block titration with approximately 5 C′ $H_{50}$ units of guinea pig complement.[12] Day-old rat liver cell suspensions were added in tenfold increments from $10^3$ to $10^6$ cells. The final volume in each reaction mixture was 1.3 ml. Veronal buffer with added $Ca^{++}$ and $Mg^{++}$ was used as diluent, and

TABLE 2*a*

COMPLEMENT FIXATION WITH DAY-OLD RAT LIVER AS ANTIGEN AND ANTI-ADULT RAT LIVER ANTISERUM ABSORBED WITH HEPATECTOMIZED DAY-OLD RAT

| As/G | $2 \times 10^6$ | $2 \times 10^5$ | $2 \times 10^4$ | Veronal buffer |
|---|---|---|---|---|
| 1/25 | 0.165 | 0.053 | 0.240 | 0.688 |
| 1/50 | 0.181 | 0.046 | 0.385 | 0.692 |
| 1/100 | 0.695 | 0.059 | 0.470 | 0.702 |
| 1/200 | 0.820 | 0.525 | 0.760 | 0.822 |
| 1/400 | 0.850 | 0.728 | 0.650 | 0.740 |
| Veronal buffer | 0.807 | 0.663 | 0.685 | 0.760 |

TABLE 2*b*

COMPLEMENT FIXATION WITH DAY-OLD RAT LIVER AS ANTIGEN AND ANTI-DAY-OLD-RAT-KIDNEY-CULTURE ANTISERUM ABSORBED WITH ADULT RAT LIVER

| As/G | $2 \times 10^6$ | $2 \times 10^5$ | $2 \times 10^4$ | Veronal buffer |
|---|---|---|---|---|
| 1/25 | 0.505 | 0.695 | 0.695 | 0.732 |
| 1/50 | 0.728 | 0.695 | 0.712 | 0.712 |
| 1/100 | 0.810 | 0.668 | 0.728 | 0.700 |
| 1/200 | 0.932 | 0.720 | 0.735 | 0.710 |
| 1/400 | 0.890 | 0.700 | 0.686 | 0.745 |
| Veronal buffer | 0.686 | 0.712 | 0.688 | 0.688 |

Antisera against adult rat liver and antiserum against cultures of day-old rat kidney were incubated wity liver cells from day-old rats, at 37°C for one hr with frequent agitation. $10^8$ sensitized sheep red cells were added and the incubation continued for another hr. Sedimentable material was removed by centrifugation and the optical density of the supernatant measured at 541 mμ.

appropriate controls included to test for anticomplementary activity at each level of antiserum and antigen. After incubation at 37°C for one hr, $10^8$ sensitized sheep red blood cells were added, and complement fixation determined by the extent of lysis of the red cells. The results are presented in Table 2, where the amount of complement fixed is in inverse relation to the optical density of the lysed erythrocytes. It is seen that anti-adult rat liver antiserum absorbed with hepatectomized day-old rat gave a typical block titration pattern with day-old rat liver. On the other hand, anti-kidney culture antiserum, absorbed with adult liver, gave perceptible complement fixation with day-old rat liver only at the highest levels of antibody and antigen that could be used without being anticomplementary.

3. *Pretreatment of inocula with antisera:* It must be emphasized that the antigenic differences between populations of parenchymal cells and tissue culture cells were, of necessity, observed only after the period of growth required to obtain a culture population. In general, this took about two weeks, and it was of interest to inquire into the mode of origin of these antigenic differences. There were at least two possibilities. First, the parenchymal liver cells of the young rat underwent antigenic modification during the *in vitro* culture period. Alternatively, the antigens present after two weeks in culture were present initially, in the inoculum,

TABLE 3

PRETREATMENT OF DAY-OLD RAT LIVER INOCULA WITH ANTISERA

| Serum | Unabsorbed | Absorbed with adult liver | Absorbed with hepatectomized day-old rat |
|---|---|---|---|
| | Number of attached and stretched cells per plate | | |
| Normal | 1850 | 3000 | 1740 |
| Antiadult liver | 1200 | 1000 | 1000 |
| Anti-day-old-rat-kidney culture | 3 | 38 | 1800 |

Fresh inocula prepared from day-old rat liver were incubated at 37°C for 30 min in the neutral synthetic medium supplemented with antisera at 5 per cent (v/v) and 3 C′ H50 units of guinea pig complement per ml. The inocula were centrifuged and washed to remove unreacted antibody and complement. An amount of tissue equivalent to $10^6$ cells were inoculated into each plate containing the standard medium. After a week of growth the plates were washed, fixed and stained, and the attached and stretched cells counted.

on cells different from the parenchymal cells and capable of growth in culture. To examine these proposals, it was planned to pretreat fresh inocula of day-old rat liver with the identical set of rabbit antisera employed in the previous experiment, then plate in the absence of antiserum. It could be predicted that if it were the parenchymal cells that were modified, then growth should be blocked by anti-liver antiserum. If, on the other hand, the antigens were there initially, then anti-culture antiserum absorbed with liver should inhibit growth. In the experiment, inocula of day-old rat liver were incubated at 37°C for 30 min in the neutral standard medium supplemented with antisera at 5 per cent (v/v), and 3 C′ $H_{50}$ units of guinea pig complement per ml. The inocula were centrifuged and washed to remove unreacted antisera and complement, and plated in the absence of antisera in the standard medium. Each plate received an inoculum equivalent to $10^6$ cells. A week later the plates were fixed and stained, and the number of attached and stretched cells per plate counted. The results presented in Table 3, demonstrate that the cells destined to give rise to the final tissue culture population had the same spectrum of antiserum sensitivity as did the final population.

4. *Ornithine transcarbamylase content of tissue culture derived from day-old rat liver:* The results obtained in the serological studies were entirely consistent with

the possibility that liver tissue culture populations originated from a cell type other than the liver parenchyma. To examine this possibility further, additional properties associated with liver parenchymal cells were investigated. It has been shown that in the rat, the enzyme, ornithine transcarbamylase, is restricted to the liver.[9] It is found in but few other tissues and there in low specific activities.** A study was made, therefore, of the ornithine transcarbamylase content of day-old rat liver and derived tissue cultures.

In this experiment, two-week old cultures, grown in the presence of ornithine (0.1 mg/ml) were harvested by the following procedure: the Petri dishes were repeatedly washed with saline to remove as much of the loose cells and pieces of tissue (nongrowing portion of inoculum) as possible. The cells were then covered with the final suspending fluid (0.9% KCl, 0.001 *M* glutathione) and scraped with a rubber policeman to remove the cells adhering to glass. This suspension was then

TABLE 4

ORNITHINE TRANSCARBAMYLASE ACTIVITY OF LIVER-DERIVED TISSUE CULTURES

| Material | Ornithine-transcarbamylase activity in micromoles of citrulline per mg protein per twenty min |
|---|---|
| Two-week-old liver cultures | 0.5 |
| Fresh day-old rat liver preparation (inoculum) | 10.0 |
| Adult rat liver (whole) | 10.0 |
| Purified single cell suspension of adult rat liver | 20.0 |

Two-week-old liver cultures were assayed for ornithine transcarbamylase activity by the method of M. E. Jones. The cells were harvested, by first washing with saline to remove loose cells and tissue. The final suspending fluid was added and the glass-attached cells removed by scraping with a rubber policeman.

ground in a Potter-Elvejhem homogenizer and the homogenate assayed for ornithine transcarbamylase. The results are presented in Table 4. The activities of whole adult liver, of a purified single cell suspension of adult liver parenchyma, and of fresh day-old rat liver preparation (the inoculum) are presented for comparison. The experiment showed that the ornithine transcarbamylase activity of liver cultures was low, with a value only 5 per cent that of the inoculum used to initiate the culture. It was found that this residual activity could be attributed to the persistence of nongrowing portions of the inoculum in spite of the strenuous efforts to wash them away. As the next experiment demonstrates, ornithine transcarbamylase does persist at high levels in nongrowing cells *in vitro*, particularly in the presence of ornithine.

5. *The persistence of ornithine transcarbamylase in nongrowing cells in vitro:* Single cell suspensions of adult liver were inoculated into standard culture medium to which ornithine at 0.1 mg/ml was added. The cells were then periodically assayed for ornithine transcarbamylase. (Under none of a wide variety of cultural conditions has an increase of cell number ever been observed with adult liver cell preparations.) Preparations of day-old rat liver were incubated in the same manner with replicate cultures from which the ornithine was omitted. Although growth was obtained with the young rat preparation, only the nongrowing, loose, and unattached cells were assayed. The results are presented in Figure 1 where it is seen that ornithine transcarbamylase persisted in nongrowing cells *in vitro* for relatively long periods. In the presence of ornithine, ornithine transcarbamylase activity in both adult and young rat liver preparations was still 50 per cent that of the inoculum at the end of two weeks. In the absence of ornithine, enzymatic

activity dropped more sharply but was still about 20 per cent that of the inoculum.

6. *The serum albumin content of liver culture cells:* Since the mammalian liver is known to be the site of serum albumin biosynthesis, it was of interest to examine liver-derived cultures for serum albumin content. Ten-day old cultures of day-old rat liver were harvested by the method used in the ornithine transcarbamylase assays. The attached and stretched cells, and the loose, nongrowing portions of the inoculum were assayed separately for serum albumin, by complement fixation with rabbit anti-rat-serum-albumin antiserum prepared by Dr. Lawrence Levine. For the assay the cells were disrupted by sonic oscillation. Freshly prepared liver from perfused day-old rats was assayed in the same manner after exhaustive washing with saline to a constant serum albumin value. The values obtained for fresh liver, nongrowing cells, and growing cells in micrograms of serum albumin per milligram of protein were 2.3, 2.7, and 0.4, respectively. The results parallel those obtained with ornithine transcarbamylase, in that the differentiated property persists in the nongrowing cells and decreases in the growing cells. Again, the residual serum albumin in the growing cells can be attributed to the persistence of portions of the inoculum.

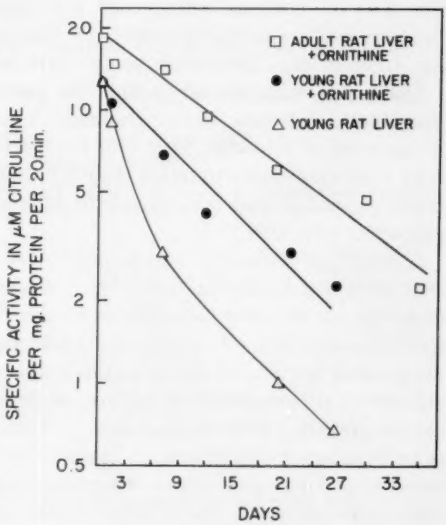


FIG. 1.—Single cell suspensions of adult rat liver parenchyma were incubated in the standard medium supplemented with 0.1 milligrams of ornithine per ml. The standard preparation of day-old rat liver was incubated in the same manner, with replicate plates from which ornithine had been omitted. From day to day the loose, unattached cells were assayed for ornithine transcarbamylase.

*Discussion.*—The major experimental findings presented in these studies are that: (1) tissue culture populations derived from day-old rat liver differ markedly in antigenic structure from day-old liver, (2) freshly prepared liver inocula are prevented from initiating culture growth by pretreatment with kidney tissue culture antiserum which has been absorbed with liver. Moreover the growth potential of liver inocula is unaffected by anti-liver antiserum containing demonstrable antibodies against liver. In addition, short term experiments reveal that liver cultures possess sharply reduced ornithine transcarbamylase activity and serum albumin content. The residual enzyme activity and albumin content can be accounted for by the microscopically demonstrable persistence of portions of the inoculum in these experiments.

We conclude from these facts that the bulk of the tissue culture population derived from day-old rat liver arises from a cell type other than the parenchymal cell. That this cell constitutes a small minority of the total cell population is supported by the finding in our laboratory, to be reported in detail elsewhere, that single cell

suspensions of day-old liver have a plating efficiency of approximately $10^{-4}$. In a forthcoming publication, evidence will be presented that this figure is a true measure of the fraction of cells of the day-old rat liver which are capable of growth in the conventional tissue culture situation.

The general lack of differentiated properties in established tissue culture strains suggests that a common basis exists for this phenomenon. It is not unreasonable that the usual methods of obtaining cultures from freshly excised tissue should selectively favor a minority, nonparenchymal cell type. The usual procedure is to start with large sections of tissue. The sections are planted in culture media which have been designed to support the growth of strains isolated in this particular manner. Since populations arise in these procedures by a mixed process of migration from the slice, and cell division, it is probable that the cell types favored should be those that are normally motile, not tightly bound in tissue structure, and which can accommodate themselves to the artificial conditions of *in vitro* growth.

Our experiments do not exclude the possibility that liver parenchymal cells, if successfully cultured, would gradually lose their differentiated properties. The recent work of Schindler, Day, and Fischer,[13] in which cloned cultures of cancerous mast cells were shown to retain their full capacity to synthesize histamine and serotonin, present at least one example of differentiated cells maintaining differentiated properties in culture.

A wealth of literature[14] exists purporting to show the growth of differentiated cells while at the same time established strains of such cells are in short supply. This disparity can probably be explained by the limitations involved in the experimental methods employed. In many cases short term experiments are used in which the nongrowing portions of the inoculum are assayed along with the growing cells of the culture for differentiated properties, while in others properties are studied which are not strictly specific to the tissue of origin. The first danger is most prominent in embryological experiments. Tissues, after a brief *in vitro* sojourn are reinjected into animals and give rise to differentiated structures corresponding to the tissue which was cultured. The conclusion that the structure was derived from differentiated cells which had multiplied in culture is suspect, since it is difficult in such studies to obtain a measure of the number of cells required to initiate the structure, and a small contamination of the cultures with nondividing but surviving portions of the inoculum could account for the results. Our studies on the persistence of ornithine transcarbamylase in nongrowing cells *in vitro* show how easily this possibility may be realized. The presence of arginase, rhodanase, and glycogen has been offered as evidence by Earle and his associates[15] that certain of their strains are derived from liver parenchyma. Although these properties are most prominent in liver, we feel that the relative degree of specificity afforded by these criteria is insufficient for a final conclusion because these properties singly and in combination have been found in tissue cultures of nonliver origin.[15–17]

The conclusion that liver cultures lack liver properties because they are descended from non-liver cells suggests that some of the difficulties in tissue culture isolation procedures can be overcome by techniques designed to suppress the growth of the contaminating cell type. Antiserum specific for this hypothetical cell type can be used in enrichment culture procedures and, in the case of liver, advantage can be taken of the fact that the liver is capable of synthesizing those nonessential amino

acids such as arginine which are found to be universally required for culture growth. These procedures are now being employed in our laboratory, but to date no survivors to anti-tissue culture serum have been found, and no cells have been isolated from liver which are arginine-independent. We feel that this may reflect a far more serious obstacle in the culture of differentiated cells. In the case of liver,[18] it is known that simple removal of liver from the body results in an immediate and drastic hydrolysis of the pyridine nucleotide coenzymes in the excised lobe. It is possible that biochemical lesions of a similar lethal effect may occur in many differentiated cells as a result of excision.

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**Ornithine transcarbamylase activity of various rat tissues, from M. E. Jones *et al.* (unpublished data):

| Tissue | O.T.C. $\mu M$ citrulline/gm. fresh tissue/hr | Tissue | O.T.C. $\mu M$ citrulline/gm. fresh tissue/hr |
|---|---|---|---|
| Liver | 4400 | Spleen | 0 |
| Intestinal mucosa | 100 | Lung | 0 |
| Kidney | 5 | Plasma | 0 |
| Salivary gland | 12 | Plasma cells | 0 |
| Brain | 0 | Skeletal muscle | 0 |
| Testis | 0 | Adrenal | 0 |
| Thymus | 0 | Thyroid | 0 |
| Pancreas | 0 | | |

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## *GENETIC CONTROL OF PHOTOSYNTHESIS IN CHLAMYDOMONAS REINHARDI**

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An increasing knowledge of the physical and chemical events which attend the process of photosynthesis opens the way to an investigation of genetic controls of this fundamental phenomenon and permits us to consider the question of the degree of control over photosynthesis exerted by the nucleus and the chloroplast.

Genetic controls of photosynthesis can be envisaged as preceding along two pathways. First, the controls can be direct. For example, there may be genetic control over the synthesis of enzymes specifically involved in photosynthetic reactions. These controls may be independent of a second pathway in which genetic changes in chloroplast structure so alter the site of photosynthesis as to affect numerous photosynthetic reactions.

There are, at present, forty-two UV-induced mutants of the sexually reproducing unicellular green alga, *Chlamydomonas reinhardi*, which cannot grow in the light unless the minimal medium is supplemented with sodium acetate. Each of the mutants represents a simple genetic change in that it segregates in a one-to-one fashion when crossed to wild-type.

The requirement for a carbon source other than or in addition to carbon dioxide suggests at least three possibilities for genetic blocks in these mutants. First, the acetate mutants may be unable to carry out certain of the light-requiring reactions of photosynthesis and thus possess genetic blocks which lie along the first pathway mentioned above. Second, the acetate mutants may have altered pigments or they may be pigment-deficient, and therefore are mutants which act indirectly on photosynthesis by way of the second pathway. Third, the mutants may possess metabolic blocks at some non-photosynthetic step in their intermediary metabolism.

One mutant strain, *acetate-21* (*ac-21*), appears to be incapable of sufficient carbon dioxide fixation to grow photosynthetically, and thus it falls into the category of a mutant strain which may be blocked in some essential step of photosynthesis.

Such a mutant provides a means for initiating an investigation into direct genetic controls of photosynthesis in sexually reproducing green algae. Accordingly, it is the purpose of the present paper to describe some of the phenomena associated with the growth and photosynthetic capabilities of *ac-21* in comparison with the wild-type strain.

*Growth Rate of ac-21.*—The growth rates of *ac-21* and wild-type were compared in both the light and the dark. *C. reinhardi* is not an obligate photoautotroph as,

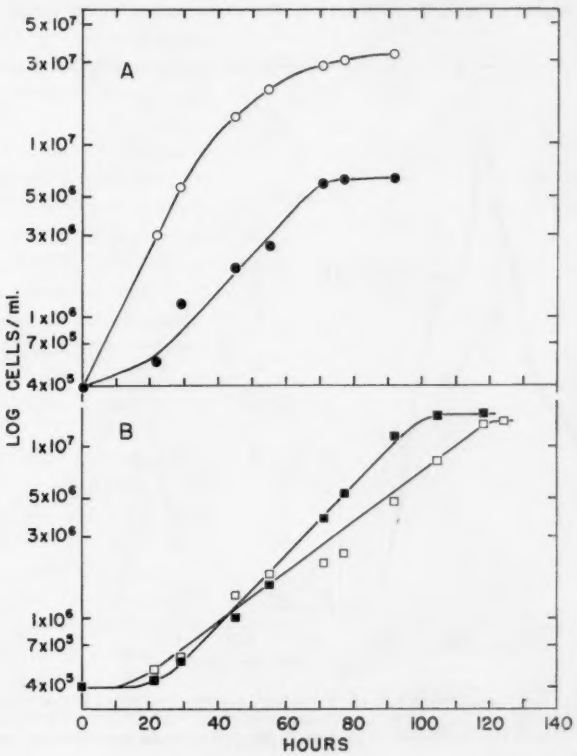


FIG. 1.—Growth of wild-type and *ac-21*. A. Growth of wild-type in the light (—○—) and in the dark (—●—). B. Growth of *ac-21* in the light (—□—) and in the dark (—■—).

for example, are the species *C. moewusii* and *C. eugametos.*[1] The wild type strain of *C. reinhardi* will grow in the dark if the minimal medium is supplemented with sodium acetate.[2]

Growth rates were measured in liquid medium in cylindrical separatory funnels 18 cm long and 5 cm in diameter. Each culture was aerated with five per cent carbon dioxide in air which also served as a source of agitation. The cultures were grown at 25°C and at a light intensity of 800 foot candles. For growth in the dark the funnels were covered with black masking tape and placed in the same

rack along with the funnels exposed to the light. Samples were withdrawn from the separatory funnels by way of the stopcock at the bottom and the measurements of growth were done by determining optical density at 750 mμ. The optical density values were converted to cells per ml according to a standard calibration curve previously obtained.

The results of a typical growth experiment are summarized in Figure 1. The growth rates, taken as the time for doubling of cell number during the logarithmic phase of growth, are as follows: wild-type (light), 8 hr; wild-type (dark), 14 hr; *ac-21* (light), 21 hr; and *ac-21* (dark), 18 hr.

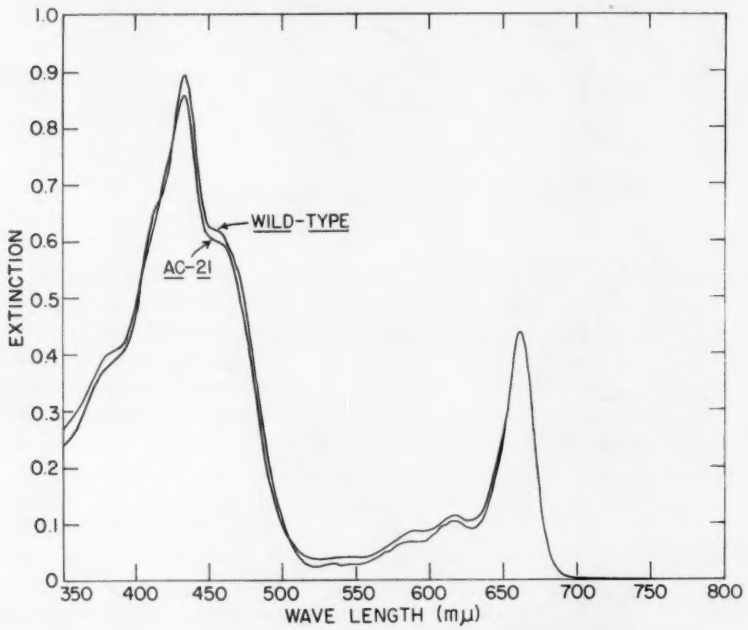


FIG. 2.—Absorption spectra of an 80 per cent acetone extract of the total pigments of wild-type and *ac-21*. The measurements were made with a Cary recording spectrophotometer and the maximum extinction in the red of *ac-21* was set equal to that of wild-type.

Unlike the growth of wild-type which is stimulated by light, the growth of *ac-21* in the light follows quite closely the curve of growth in the dark. For reasons not yet fully understood, wild-type grown in the dark reaches the stationary phase somewhat earlier than *ac-21*. This may be an artifact due to differences in cell size which would affect optical density readings, or may be due to a greater limiting effect of acetate concentration on the growth of wild-type in the dark.

*The Pigments of ac-21.*—The inability of *ac-21* to grow in the light in the absence of acetate might lie in an alteration of either the composition or concentration of its pigments. Numerous determinations were made of the amount of chlorophyll for both wild-type and *ac-21* using the method of Arnon.[3] Each chlorophyll determina-

tion was accompanied by a determination of cell number and the cells used were from log-phase cultures. The values for $\mu$g chlorophyll per $10^6$ cells in a series of four independent cultures of wild-type and of *ac-21* were as follows: wild-type—1.87, 1.40, 1.95, and 1.17; *ac-21*—1.69, 1.75, 1.71, and 1.07. The averages for wild-type and *ac-21* are, respectively, 1.68 and 1.62. There is, therefore, no marked quantitative difference between wild-type and *ac-21* with respect to the amount of chlorophyll per cell.

In addition, a comparison of the spectra from a total pigment extract reveals little, if any, qualitative variation between wild-type and *ac-21* (Fig. 2). Furthermore, electron micrographs have revealed that chloroplast structure is identical in wild-type and *ac-21*.[4]

*The Hill Reaction.*—Using parabenzoquinone as a hydrogen acceptor, the Hill reaction of whole cells was measured in phosphate buffer for both wild-type and *ac-21* which had been obtained from log-phase cultures. The measurements were carried out in duplicate for wild-type and *ac-21* in Warburg flasks in an atmosphere of nitrogen at 15°C and at a light intensity of 400 foot candles. The amount of chlorophyll per flask was 0.2 mg for both wild-type and *ac-21*. The $Q_{O_2}^{Chl}$ (calculated as microliters oxygen liberated per hr per mg chlorophyll) was 750 for wild-type and 546 for *ac-21*. These results were similar to those obtained in two additional experiments. Thus, under the conditions of the experiment described here, *ac-21* has a Hill reaction which is 73 per cent as effective as that of wild-type.

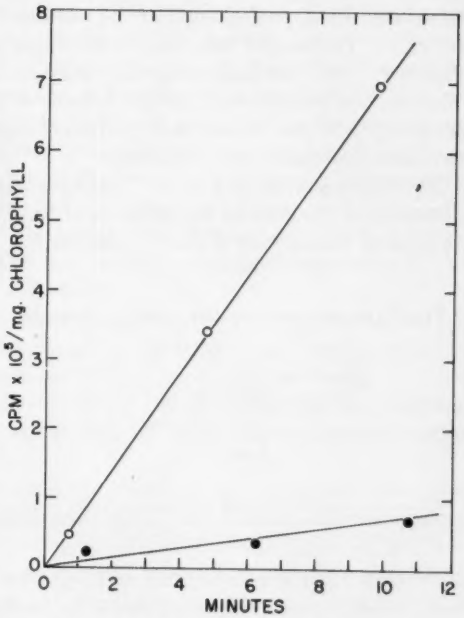


FIG. 3.—The rates of carbon dioxide fixation of wild-type (—○—) and *ac-21* (—●—). The values have been corrected for the amount of carbon dioxide fixation in the dark.

*The Rate of Carbon Dioxide Fixation.*—The rate of carbon dioxide fixation in whole cells of wild-type and *ac-21* was determined in a series of three experiments. The age of the cells and conditions of temperature and light were as for the measurement of the Hill reaction. The measurement of carbon dioxide fixation was carried out in the light and dark in 25 ml Erlenmeyer flasks stoppered with serum caps. For both wild-type and *ac-21* one flask was exposed to light while the other was covered with black masking tape. Washed cells, which were suspended in buffer, were allowed to equilibrate for 10 min before carbon-14 labeled sodium bicarbonate was added. The lights were turned on after the addition of the sodium bicarbonate. Samples were removed with a syringe at the times indicated in Table 1

TABLE 1

CARBON DIOXIDE FIXATION BY WILD-TYPE AND *ac-21*

| Wild-Type (cpm × $10^5$/mg Chl) | | | | *ac-21* (cpm × $10^5$/mg Chl) | | | |
|---|---|---|---|---|---|---|---|
| Time (min) | Light | Dark | Light minus dark | Time (min) | Light | Dark | Light minus dark |
| 0.75 | 0.59 | 0.11 | 0.48 | 1.25 | 0.36 | 0.13 | 0.23 |
| 4.75 | 3.60 | 0.20 | 3.40 | 6.25 | 0.58 | 0.21 | 0.37 |
| 9.75 | 7.23 | 0.26 | 6.97 | 10.75 | 0.97 | 0.26 | 0.71 |

Each reaction mixture contained either wild-type or *ac-21* (0.2 mg Chlorophyll), 4.7μmoles of $NaHC^{14}O_3$ (specific activity, 0.76 μc/μmole) and phosphate buffer (pH 6.8).

and Figure 3. Each sample was run into 0.06 ml of 0.5 *M* hydrochloric acid in order to stop fixation. Aliquots of each sample were plated in duplicate to weighed planchets, dried, reweighed, and then counted to determine the amount of radioactivity. The results from one of the experiments are shown in Table 1 and in Figure 3. They are the average for duplicate samples corrected for self-absorption, dilution, and background and are typical of those obtained in the other two experiments. It can be seen that the rate of carbon dioxide fixation in *ac-21* is some ten times less than it is in wild-type.

*The Carboxylation of Ribulose 1,5-Diphosphate.*—Fixation of carbon dioxide was measured in the dark in the presence of ribulose 1,5-diphosphate according to the method of Smillie and Fuller.[5] The results, which are summarized in Table 2,

TABLE 2

THE CARBOXYLATION OF RIBULOSE 1,5-DIPHOSPHATE (RuDP), BY WILD-TYPE AND *ac-21*

| | RuDP | $C^{14}O_2$ Fixed (cpm/mg Chl/hr) |
|---|---|---|
| Wild-type | + | $0.43 \times 10^5$ |
| | − | $0.14 \times 10^5$ |
| *ac-21* | + | $1.07 \times 10^5$ |
| | − | $0.11 \times 10^5$ |

The reaction mixture contained in addition to either wild-type or *ac-21* (0.2 mg of chlorophyll) which had been disrupted in a Mullard ultrasonicator, 1μmole of ribulose 1,5-diphosphate, 4.7 μmoles of $NaHC^{14}O_3$ (specific activity 0.76 μc/μmole), and phosphate buffer (pH 6.8).

show that both wild-type and *ac-21* possess an active ribulose 1,5-diphosphate carboxylase. Since the experiments as performed here are still in a very preliminary stage, it is difficult to draw conclusions regarding the significance of the difference that appears to exist between wild-type and *ac-21*. This difference, however, has been seen in four replicate experiments.

*Photosynthetic Phosphorylation.*—Since *ac-21* liberated oxygen as measured by the Hill reaction, the electron transfer system up to this stage of photosynthesis is intact. This suggested that the genetic block might be in the step of photosynthetic phosphorylation. The results of measurements of photosynthetic phosphorylation are given in Table 3. It can be seen that the rate of photosynthetic phosphorylation of the mutant is some twelve times less than that of the wild-type. However, the difference in phosphorylation shown by the mutant in the light and the dark is negligible when the counting error is taken into consideration. Therefore, it appears that the mutant has little, if any, photosynthetic phosphorylation.

*Discussion.*—The mutant, *ac-21*, is not capable of photosynthetic growth. It cannot grow unless the minimal medium has been supplemented with sodium acetate. Furthermore, unlike wild-type, its growth rates in the light and dark are

TABLE 3

PHOTOSYNTHETIC PHOSPHORYLATION OF WILD-TYPE AND *ac-21*

| | $P^{32}$ Esterified (cpm/mg Chl/hr) | | |
|---|---|---|---|
| | Light | Dark | Light minus dark |
| Wild-type | 133,846 | 52,548 | $8.13 \times 10^4$ |
| *ac-21* | 84,150 | 77,520 | $0.66 \times 10^4$ |

The reaction mixture contained either wild-type or *ac-21* which had been disrupted in a Mullard ultrasonicator and each of the following in $\mu$ molar amounts: Tris, 12; $MgCl_2$, 6; inorganic phosphate 2.4; ascorbate, 0.6; cysteine HCl, 0.6; adenosine diphosphate, 3; flavine mononucleotide, 0.06; and vitamin $K_3$, 0.0015. The reaction was carried out for 10 min in nitrogen at 15°C at a light intensity of 400 ft. c. The esterification of phosphate was measured according to the technique of Lindberg and Ernster.[8]

essentially identical. In addition, the rate of carbon dioxide fixation in *ac-21* is some ten times less than that of wild-type. This reduced rate of carbon dioxide fixation cannot be accounted for solely on the basis of a genetic block to electron transfer, since even though the rate of oxygen liberation is reduced in the mutant to 73 per cent of that found in wild-type, this reduction is insufficient to explain the tenfold decrease of carbon dioxide fixation. In addition, the preliminary experiments reported here have shown that both wild-type and *ac-21* can bring about the fixation of carbon dioxide in the dark in the presence of ribulose 1,5-diphosphate indicating the presence of a ribulose 1,5-diphosphate carboxylase such as is found in other photosynthetic organisms. The fact that the carboxylation reaction occurs in *ac-21* suggests that the genetic block does not lie in this dark reaction leading to carbon dioxide fixation.

It is clear, however, that *ac-21* has lost the ability to carry out photosynthetic phosphorylation. The loss of this phase of its photosynthesis, even though the light-dependent oxygen evolution is unaffected, suggests that the energy provided by photosynthetic phosphorylation is required for carbon dioxide fixation unless the appropriate carbon dioxide acceptor is supplied. This is in agreement with recent suggestions made by Arnon.[6]

It can be concluded that *ac-21* is a genetic alteration at a locus which controls the process of photosynthetic phosphorylation. A mutation at this locus may result in either a genetic uncoupling of photosynthetic phosphorylation or a disruption of electron transfer below the level of the release of molecular oxygen. An alternative still to be investigated is the possibility of a genetic block affecting acceptors of phosphate from adenosine triphosphate.[9]

The demonstration that at least one of the steps in photosynthesis is under genetic control opens the way to further investigations into the role of the nucleus in determining functions which occur in the chloroplast. Additional mutants which do not fix carbon dioxide have been obtained,[7] and they are now under investigation in order to determine the nature of their blocks to photosynthesis. The genetic analysis of the mutants is also under way. Both the genetic analyses and the functional tests of these mutants may shed light on the over-all organization of nuclear controls of photosynthesis.

The suggestions and criticisms of Drs. N. I. Krinsky, W. R. Sistrom, and J. A. Schiff during the course of this work are gratefully acknowledged. The ribulose 1, 5-diphosphate was kindly supplied by Dr. E. Racker.

* This work was supported by grants from the National Science Foundation and the U.S. Public Health Service.

[1] Lewin, J. C., *Science*, **112**, 652 (1950).

[2] In the experiments reported here, cultures were grown on minimal medium plus sodium acetate (0.2 gm per 100 ml). The minimal medium, described in Levine, R. P., and Ebersold, W. T., *Zeit. Vererbungs.*, **89**, 632 (1958), was altered by a fivefold increase in the concentration of Beijerinck's solution.

[3] Arnon, D. I., *Plant Physiol.*, **24**, 1 (1949).

[4] The author is indebted to Miss Jana Moravkova and Dr. Ian Gibbons for several electron micrographs of Chlamydomonas chloroplasts.

[5] Smillie, R. M., and Fuller, R. C., *Plant Physiol.*, **34**, 651 (1960).

[6] Arnon, D. I., *Nature*, **184**, 10 (1959).

[7] Levine, R. P., *Nature* (in press).

[8] Lindberg, O., and Ernster, L., in *Methods of Biochemical Analysis*, ed. D. Glick (New York: Interscience Publ., 1956), vol. 3, p. 1.

[9] Note added in proof: Subsequent experiments have shown that extracts of *ac-21* prepared by sonication are capable of carbon dioxide fixation in the light in the presence of adenosine triphosphate.

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## *THE FACTORIZATION OF CYCLIC REDUCED POWERS BY SECONDARY COHOMOLOGY OPERATIONS**

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Steenrod[1] has defined the cyclic reduced powers

$$P^j\colon H^m(X; Z_p) \to H^{m+2j(p-1)}(X; Z_p) \tag{1}$$

for any topological space $X$ and nonnegative integer $j$. The $P^j$ are linear mappings, stable under suspension and natural in $X$. Furthermore, the algebra $A$ of all stable cohomology operations over $Z_p$ is generated by linear combinations of the compositions of $P^j$ and $\Delta$, where the Bockstein

$$\Delta\colon H^m(X; Z_p) \to H^{m+1}(X; Z_p) \tag{2}$$

is associated with the exact coefficient sequence

$$0 \to Z_p \to Z_{p^2} \to Z_p \to 0.$$

Adem[2] and Cartan[3] have studied relations between products of $P^j$'s and $\Delta$'s. It turns out that if $j$ is not a power of $p$, $P^j$ can be expressed as a linear combination of compositions $P^{i_1} \ldots P^{i_n}$, where $i_\nu < j$, $\nu = 1, \ldots, n$. That is, in the Steenrod algebra $A$ the elements $P^j$, $j \neq p^r$, are decomposable.[2]

Associated with the Steenrod algebra $A$ over $Z_p$ are certain stable secondary cohomology operations.[4] We prove that there exist stable secondary cohomology operations $\mathfrak{R}$, $\Psi_k$ ($k = 1, 2, \ldots$) such that $\mathfrak{R}$ is defined on classes $u \in H^m(X; Z_p)$ with $\Delta u = 0$, $P^1 u = 0$, and $\mathfrak{R}(u)$ is a coset of the group

$$P^2 H^m(X; Z_p) + ({}^1/_2 \Delta P^1 - P^1\Delta) H^{m+2p-2}(X; Z_p) \tag{3}$$

in $H^{m+4(p-1)}(X; Z_p)$; the operations $\Psi_k$ ($k > 0$) are defined on classes $v \in H^m(X;$

$Z_p$) such that $\Delta v = 0$, $P^{p^i}v = 0$, $(i = 0, 1, \ldots, k)$, and $\Psi_k(v)$ is a coset of

$$P^{p^k}H^m(X; Z_p) + \sum_{i=0}^{k} \vartheta_i H^{m-1+2p^i(p-1)}(X; Z_p) \tag{4}$$

in the group $H^{m+2p^k(p-1)}(X; Z_p)$, where $\vartheta_i$ are homogeneous elements of the Steenrod algebra $A$ with odd gradings.

THEOREM 1. *Let $k$ be a nonnegative integer. There exists a constant $\nu_k$, nonzero in $Z_p$, elements $a_{k,i}$, $b_k$, $c_{k,\gamma}$ of positive grading in the Steenrod algebra, and secondary cohomology operations $\Gamma\gamma$ of odd degree, such that*

$$\{\nu_k P^{p^{k+1}}\} = \sum_{i=1}^{k} a_{k,i}\Psi_i + b_k\Re + \sum_{\gamma} c_{k,\gamma}\Gamma_\gamma, \tag{5}$$

*the relation holding modulo the total indeterminacy of the right-hand side.*

That such a relation exists, with the scalar $\nu_k$ possibly zero, is an immediate consequence by the methods of J. F. Adams[4] of the following theorem.

THEOREM 2. *The vector spaces $Ext_A{}^i(Z_p, Z_p)$ for $i = 1, 2$ have the following $Z_p$ bases:*

(1) *$Ext_A{}^1(Z_p, Z_p)$ has as basis certain classes $h_i$ $(i = 0, 1, \ldots)$ of grading $2p^i(p - 1)$, $\alpha_0$ of grading 1;*

(2) *a $Z_p$ basis for $Ext_A{}^2(Z_p, Z_p)$ is furnished by certain classes $h_ih_j$, $i < j - 1$ $(i = 0, 1, \ldots, j = 2, 3, \ldots)$ of grading $2(p - 1)(p^i + p^j)$, $h_i\alpha_0$ $(i \neq 0)$ of grading $2p^i(p - 1) + 1$, $\mu_i$ of grading $2(p - 1)(p^{i+1} + 2p^i)$, $\nu_i$ of grading $2(p - 1)(2p^{i+1} + p^i)$, $\lambda_i$ of grading $2(p - 1)(p^{i+1})$, $\rho$ of grading $4(p - 1) + 1$, and $\alpha_0\alpha_0$ of grading 2;*

(3) *the elements $\lambda_j\alpha_0$ in $Ext_A{}^3(Z_p, Z_p)$ of grading $2p^{j+1}(p - 1) + 1$ are nonzero.*

The theorem is proved by using the Adams spectral sequence for Hopf algebras. The proof is made easier by introducing Steenrod operations. These operations are defined on $Ext_G{}^{r,s}(Z_p, Z_p)$, where $G$ is a graded, connected Hopf algebra over $Z_p$ with associative product and associative and commutative diagonal:

$$\begin{aligned} P^j&: Ext_G{}^{r,s}(Z_p, Z_p) \to Ext_G{}^{r+2j(p-1),ps}(Z_p, Z_p), \\ \Delta&: Ext_G{}^{r,s}(Z_p, Z_p) \to Ext_G{}^{r+1,ps}(Z_p, Z_p). \end{aligned} \tag{6}$$

The operations have most of the properties of the usual Steenrod cohomology operations. In particular, the Adem relations[2] and the Cartan formula for cup products[5] are valid. A departure from the topological case appears in the shift of the second grading. $P^0$ is no longer the identity operation:

$$\begin{aligned} P^0h_i &= h_{i+1}, \\ \Delta h_i &= \lambda_i. \end{aligned} \tag{7}$$

Let $\alpha\epsilon H^2(Z, 2; Z_p)$ be the fundamental cohomology class. The indeterminacies involved in (5) vanish for $H^*(Z, 2; Z_p)$, thus the relation becomes a relation on classes, not cosets. We find the unknown coefficient $\nu_k$ in Theorem 1 by evaluating the relation (5) on the class $\alpha^{p^{k+1}}$. For this, we need to know the values of $\Re$ and $\Psi_k$ on $\alpha^{p^{k+1}}$.

THEOREM 3. *Let $t$ be a positive integer. There exist nonzero constants $b$, $c_k$ in $Z_p$ such that*

$$\Re(\alpha^{p^t}) = b\iota\alpha^{p^t+2(p-1)}, \tag{8}$$

$$\Psi_k(\alpha^{p^{k+1}t}) = c_k t\ \alpha^{p^{k+3}t+p^k(p-1)}. \tag{9}$$

The theorem is proved by considering the cohomology of universal example spaces associated with a minimal resolution of $Z_p$ over $A$. Since these spaces can be taken to be iterated loop spaces, both the homology and cohomology are Hopf algebras over $Z_p$ with commutative associative product and diagonal. The universal examples for $2p^{k+1}$-dimensional classes turn out to be homotopically equivalent to Cartesian products of $K(Z_p, n)$'s, but not as loop spaces. In cohomology this is reflected by the decomposition of the cohomology into a tensor product as algebras, but not as coalgebras. It turns out that primitive elements defining the operations $\mathcal{R}$, $\Psi_k$ can be completely described, enabling us to evaluate $\Psi_k(\alpha^{p^{k+1}})$ and $\mathcal{R}(\alpha_p)$. The values of $\Psi_k(\alpha^{p^{k+1}t})$ and $\mathcal{R}(\alpha^{pt})$ ($t$ a nonzero integer) are then found by using the Adams formula for secondary operations on cup products.[4]

At a critical point in the proof, a consequence of a theorem by W. Browder[6] is used strongly. Let

$$\sigma_{*n}\colon H_n(\Omega X; Z_p) \to H_{n+1}(X; Z_p)$$

be the homology suspension.

THEOREM 4. *If $X$ is an $H$-space, then Kernel $\sigma_{*n}$ is precisely the set of decomposable elements of $H_n(\Omega X; Z_p)$ if $n$ cannot be written in the form $(2m)p^f - 2$, where $f$ and $m$ are integers.*

Theorem 1 is related to the question of the existence of elements of mod $p$ Hopf invariant one. The mod $p$ Hopf invariant is a homomorphism

$$\gamma_{p,i}\colon \Pi_{m+n}(S^m) \to Z_p, \tag{10}$$

where $n = 2i(p-1) - 1$, defined as follows: given a class $\alpha$ in $\Pi_{m+n}(S^m)$, pick an element $f$ of $\alpha$. Define the cell complex $K_f = S^m \cup_f E^{m+n}$ by attaching an $(m+n)$-cell to $S^m$ by $f$. Pick standard generators $\iota'_0$, $\iota'_m$, $\iota'_{m+n}$ for the three nonzero groups of $H^*(K_f; Z_p)$. Then $\gamma_{p,i}(\alpha)$ is defined by

$$P^i\iota'_m = \gamma_{p,i}(\alpha)\iota'_{m+n}. \tag{11}$$

Adem[2] proved that $\gamma_{p,i}$ is zero, unless $i = p^r$, for some nonnegative integer $r$. Borel and Serre[7] and Toda[8] proved that $\gamma_{p,1}$ is nontrivial. Milnor[9] showed that $\gamma_{p,i}$ is zero on the image of the Whitehead $J$-homomorphism if $i > 1$. These results are contained in the following immediate consequence of Theorem 1.

THEOREM 5. *If $i > 1$, then $\gamma_{p,i}$ is identically zero.*

An immediate result of this (for $p = 3$) is [8, 10] the following theorem.

THEOREM 6. *Let $\iota_{2n}$ be the homotopy class of the identity map in $\Pi_{2n}(S^{2n})$, and let*

$$[\iota_{2n}, [\iota_{2n}, \iota_{2n}]] \tag{12}$$

*be the iterated Whitehead product. Then if $n > 1$, the element $[\iota_{2n}, [\iota_{2n}, \iota_{2n}]]$ is an element of order 3 in $\Pi_{6n-2}(S^{2n})$.*

* The study here summarized was done while the author held a National Science Foundation Fellowship.

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# *NOTE ON POLYNOMIAL APPROXIMATION ON A JORDAN ARC**

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*Communicated May 23, 1960*

In two recent notes[1, 2] the present writer has discussed the invariance of degree of polynomial and trigonometric approximation under change of independent variable. However, those notes did not emphasize approximation in the complex plane on a Jordan arc rather than on a Jordan curve; the object of the present note is to indicate rapidly additional results, concerning Jordan arcs.

We state for reference

THEOREM 1. *Let $\epsilon_1, \epsilon_2, \ldots$ be a sequence of positive numbers approaching zero, where we suppose $\epsilon_{[n/\lambda]} = O(\epsilon_n)$ for every positive integral $\lambda$ (here $[m]$ denotes the largest integer not greater than $m$), and where for every $r$, $0 < r < 1$, we have $r^n = O(\epsilon_n)$. Let $E$ be a Jordan arc in the $z$-plane, and let $D$ be a region containing $E$. Let the function $f(z)$ be defined on $E$, let the functions $f_n(z)$ be analytic in $D$, and suppose ($n = 1, 2, 3, \ldots$)*

$$|f(z) - f_n(z)| \leqq A_1\epsilon_n, \qquad z \text{ on } E, \tag{1}$$

$$|f_n(z)| \leqq A_2R^n, \qquad z \text{ in } D. \tag{2}$$

*Then there exist polynomials $p_n(z)$ in $z$ of respective degrees $n$ such that*

$$|f(z) - p_n(z)| \leqq A_3\epsilon_n, \qquad z \text{ on } E. \tag{3}$$

Here and below, the letter $A$ with subscript denotes a positive constant independent of $n$ and $z$.

Theorem 1 is contained in the comments to the Corollary of Theorem 1 in reference 1. Indeed, it is not necessary that $E$ be a Jordan arc; it is sufficient if $E$ is a closed bounded set.

If $E$, $D$, and $f(z)$ satisfy the hypothesis of Theorem 1 (not including inequalities (1) and (2)), if $D$ is bounded, and if inequality (3) is valid for polynomials $p_n(z)$ of respective degrees $n$, then inequality (2) with $f_n(z)$ replaced by $p_n(z)$ is also valid if $R$ is suitably chosen. That is to say, if $D$ is bounded, inequality (3) is not merely necessary but also sufficient for inequalities (1) and (2). Indeed, inequality (3) implies $|p_n(z)| \leqq A_4$, $z$ on $E$, which by the generalized Bernstein Lemma (§4.6 of reference 3) implies $|p_n(z)| \leqq A_4R^n$, $z$ on $E_R$, where $E_R$ is the level locus $g(z) = \log R$ ($>0$) of Green's function $g(z)$ for the complement of $E$ with pole at infinity. For sufficiently large $R$, $D$ lies interior to $E_R$.

The particular interest of Theorem 1 lies largely in the fact that the hypothesis

(and hence the conclusion) is invariant under one-to-one conformal transformation of $E$; such a transformation is also one-to-one and conformal in a suitably chosen subregion of $D$ containing $E$, and the transforms of the $f_n(z)$ possess the properties required by Theorem 1 in the image of this subregion. Thus a direct application of Theorem 1 is

THEOREM 2. *Under the conditions of Theorem* 1, *let* $E$ *be an analytic Jordan arc, and let* $E$ *be mapped one-to-one and conformally onto the arc* $E_1$ *of the* $w$*-plane, with* $f(z)$ *transformed into* $f_1(w)$. *Then on* $E_1$ *the function* $f_1(w)$ *can be approximated by polynomials in* $w$ *of respective degrees* $n$ *with degree of approximation* $O(\epsilon_n)$. *Thus the class of functions* $f_1(w)$ *which can be approximated on* $E_1$ *by polynomials in* $w$ *with degree of approximation* $O(\epsilon_n)$ *is identical with the class of transforms of functions* $f(z)$ *which can be approximated on* $E$ *by polynomials in* $z$ *with degree of approximation* $O(\epsilon_n)$, *which is identical with the class of transforms (by a conformal map) of functions* $F(x)$ *which can be approximated on* $E_0$: $-1 \leqq x \leqq 1$ *by polynomials in* $x$ *of respective degrees* $n$ *with degree of approximation* $O(\epsilon_n)$.

With $\epsilon_n = 1/n^{p+\alpha}$, where $p$ is a nonnegative integer and $0 < \alpha \leqq 1$, it is a consequence of the Bernstein-Jackson-Montel-de la Vallée Poussin-Zygmund theory of trigonometric approximation that $F(x)$ can be approximated on $E_0$ by polynomials in $x$ with degree of approximation $O(\epsilon_n)$ when and only when $F(\cos\theta)$ is a function of $\theta$ whose $p$th derivative (with respect to $\theta$) satisfies in $\theta$ a Lipschitz condition of order $\alpha (0 < \alpha < 1)$ or Zygmund condition ($\alpha = 1$). It follows by Theorem 2 that $f(z)$ *can be approximated on* $E$ *by polynomials in* $z$ *with degree of approximation* $O(n^{-k-\alpha})$ *when and only when* $f[\phi(\cos\theta)]$ *is a function of* $\theta$ *whose* $p$*th derivative (with respect to* $\theta$*) satisfies a Lipschitz condition of order* $\alpha (0 < \alpha < 1)$ *or Zygmund condition* ($\alpha = 1$); *here* $z = \phi(x)$ *maps* $E_0$ *one-to-one and conformally onto* $E$.

The remark just made is closely related to Theorem 4 of reference 4. The following result is likewise closely related to Theorem 5 and the comments (not proved in detail) to Theorem 4, both of reference 4:

THEOREM 3. *If the* $\epsilon_n$ *satisfy the conditions of Theorem 1, and if* $E$ *is an analytic Jordan arc of the* $z$*-plane on which the function* $f(z)$ *is defined, then* $f(z)$ *can be approximated on* $E$ *by polynomials in* $z$ *with degree of approximation* $O(\epsilon_n)$ *when and only when the function* $f(z) \equiv f_2(\mathcal{I}\cos\theta_1) \equiv f_2(s)$ *can be so approximated by trigonometric polynomials in* $\theta_1$; *here* $2\mathcal{I}$ *is the length of* $E$ *and* $s$ *is arc-length on* $E$ *measured along* $E$ *algebraically from the mid-point of* $E$.

Theorem 3 is analogous to a result[1, 2] concerning invariance under conformal mapping of degree of approximation on the unit circle $|z'| = 1$ by polynomials in $z'$ and $1/z'$, that is to say, by trigonometric polynomials in $\theta' = -i \log z'$.

To prove Theorem 3 it is sufficient (Theorem 2 of reference 2) to show that under the transformation $z = z(s) \equiv z(\mathcal{I}\cos\theta_1) = \phi(\cos\theta) \equiv \phi(x)$ the function $\theta_1$ is an analytic function of $\theta$ with nonvanishing derivative, a derivative that clearly is different from zero if $\cos\theta \neq \pm 1$. We choose $\mathcal{I} = 1$, whence $s = \cos\theta_1$, so from $x = \cos\theta$ follows for sufficiently small $|x - 1|$

$$s = \cos\theta_1 = a_0 + a_1(x-1) + a_2(x-1)^2 + \ldots, \quad a_1 \neq 0.$$

Under the transformation used we may suppose that $x = 1$ corresponds to $s = 1$, whence $a_0 = 1$ and $\cos\theta_1$ is

$$1 - \frac{\theta_1^2}{2!} + \frac{\theta_1^4}{4!} - \ldots = 1 + a_1\left(-\frac{\theta^2}{2!} + \frac{\theta^4}{4!} - \ldots\right) + a_2\left(-\frac{\theta^2}{2!} + \frac{\theta^4}{4!} - \ldots\right)^2 + \ldots.$$

This relation is equivalent to

$$\theta_1^2 = a_1\theta^2 + b^2\theta^4 + \ldots,$$

and with proper attention to algebraic signs,

$$\theta_1 = a_1^{1/2}\theta + c_2\theta^2 + \ldots.$$

Thus $\theta_1$ is an analytic function of $\theta$ with nonzero derivative in the neighborhood of $\theta = 0$. A corresponding discussion near $x = -1$ yields the analyticity of $\theta_1$ as a function of $\theta$ with nonzero derivative in the neighborhood of $\theta = \pi$, and this completes the proof of Theorem 3.

It is a consequence of Theorem 3 that a necessary condition that $f(z)$ can be approximated on $E$ by polynomials in $z$ of respective degrees $n$ with degree of approximation $O(n^{-k-\alpha})$, $0 < \alpha < 1$, is that on any closed subsegment of $E$ not containing an end point $f_2(I\cos\theta_1)$ should have a $k$th derivative satisfying a Lipschitz condition in $\theta_1$ of order $\alpha$; this condition is sufficient except for auxiliary conditions in the neighborhoods of the end points.

Moreover, de la Vallée Poussin has established certain sufficient conditions for trigonometric approximation of various orders, depending on the modulus of continuity of the given function or of its $k$th derivative; he has also established results in the reverse direction. Thanks to Theorem 3, both kinds of results apply to the study of approximation to a function $f(z)$ on $E$, where we may apply the conditions of de la Vallée Poussin directly to the function $f(z) \equiv f_2(I\cos\theta_1) \equiv f_2(s)$ rather than to the function $f[\phi(\cos\theta)]$ which involves a conformal map of $E$ onto $E_0$: $-1 \leqq x \leqq 1$.

A similar comment applies also to approximation in the mean as measured by an integral over an analytic Jordan arc with an appropriate weight function.

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[2] Walsh, J. L., these PROCEEDINGS, **45**, 1528–1533 (1959).

[3] Walsh, J. L., *Interpolation and Approximation*, vol. 20 of Colloquium Series of Am. Math. Soc. (1935).

[4] Walsh, J. L., these PROCEEDINGS, **37**, 821–826 (1951).

# *OXYGEN AND NITRIC OXIDE AS MODIFIERS OF RADIATION INJURY IN SPORES OF BACILLUS MEGATERIUM**

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Among recent observations on the oxygen effect in radiation biology, the one that the gas nitric oxide (NO) appears to be able to substitute for oxygen in enhancing the effects of ionizing radiations in some biological systems is of particular importance. This observation was made originally by Howard-Flanders in bacteria,[1] and was later confirmed by Kihlman[2] for chromosome breaks in *Vicia* and by Gray *et al.*[3] for lethal effects in ascites tumor cells. In a more recent paper, Howard-Flanders and Jockey[4] claim molecule for molecule substitution of NO for $O_2$, and suggest that the mode of action of both substances is the same, probably the reaction of the gases with radiation-induced radicals to form a reaction product lethal to the cell.

Observations that appear to be contrary have been recorded. NO *reduces* radiation sensitivity of dry seeds of *Agrostis* (Sparrman *et al.*[5]), and of dry bacterial spores (Powers *et al.*[6]). Since the latter report,[6] we have extended our studies and in this communication shall give evidence that the action of NO in our dry bacterial system is a double one—enhancing and protecting. In addition, extension of the studies previously reported[7] on the action of oxygen are included herein. All of our results with NO and $O_2$ lead to an explanation of their actions in this single dry system that constitutes for us the basis of a general explanation of the oxygen effect.

*Methods.*—The spores were those of a nonlysogenic strain of *Bacillus megaterium* (ATTC #8245). This strain sporulates very well, yielding 100 per cent spore suspensions when grown on potato extract agar. The spores were mounted on cellulose membrane filters (Millipore filters) in our usual manner,[8] dried *in vacuo*, and exposed to X-rays in gastight containers of either of two types. In some instances, cylindrically shaped plastic containers with a 0.08-mm thick plastic window were used. In others, the containers were gastight stainless steel cylinders attached to the X-ray tube with a manifold connected to pumps and gas sources. The temperature under different exposure conditions was measured with thermocouples as explained before.[7] After the experimental treatment the filters were placed on paper pads saturated with a buffered peptone-glucose medium and incubated 16 hr at 35°C. The ability of the spores to form visible colonies was the criterion of survival. The X-ray source was a Machlett OEG 60 tube with a tungsten target and beryllium window, operated at 50 kv and 45 ma (HVL 0.070 mm Al) with no added filtration except for the thin plastic window of the gas chamber. The filters were irradiated in stacks of five at a dose rate of about 15,000 r/min in the steel gas chambers. The dose rate was 43,310 r/min in the plastic chambers.

The chambers containing the filters were pumped to less than 1 mm Hg, and then $O_2$-free $N_2$ (or at times He) was introduced to one atmosphere. This process was repeated twice. Then $N_2$ (or at times He) was introduced to one-half atmosphere, NO or $O_2$ was allowed to flow to the pressure proper for the concentration

finally desired, and $N_2$ (or He) was added to bring the pressure to one atmosphere at room temperature. When the NO or $O_2$ concentrations were very low, two chambers in series were used, the first containing $N_2$ and a certain concentration of NO at one atmosphere so that 10 per cent of this gas mixture could be passed into the second chamber containing the spores, thereby increasing the sensitivity. After irradiation or other treatment, the pumping series was reversed to remove all gas before the introduction of air, a necessary procedure since NO can combine with $O_2$ to form undesirable compounds. None of the gas or temperature changes affected the survival of control spores.

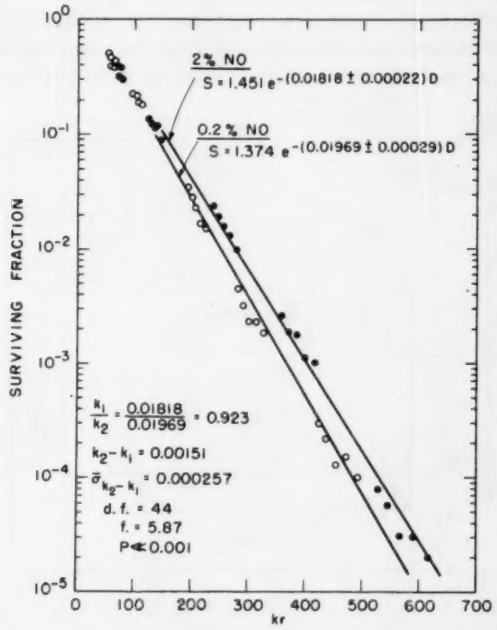


FIG. 1.—Inactivation of dry spores of *Bacillus megaterium* exposed to soft X-rays in two concentrations of nitric oxide.

As in all other studies, we utilized the slope of the survival curve as the measure of radiation sensitivity, and examined the changes in slope with changes in experimental conditions. The slope and its estimated error are calculated from

$$\text{Fraction surviving} = ne^{-kD}.$$

This relation is accurate numerically for all surviving fractions below 50 per cent. In this equation, $k$, the slope of the line, is our measure of radiation sensitivity and is expressed as reciprocal kiloroentgens, $D$ is the dosage, and $n$ is a constant that is numerically equal to the Y-axis intercept of the extrapolated, nearly linear portion of the survival curve; it is the quantity that is called the "hit-number" in many studies other than the ones in this series. We utilize this relation in a formal way only for convenience, and intend no interpretations by its use. The value of $n$ does not vary systematically with any of the treatments used but rather shows a small, apparently random, variation about a mean value of 1.30.

Usually $k$ was estimated from a survival curve consisting of from 4 to 5 points, with each point based on 5 filters. The number of colonies on each filter varied from 20 to 300, usually about 60. Therefore, each point was based, on the average, on a count of about 300 colonies divided among 5 units.

*Results.*—Typical survival curves are demonstrated in Figure 1. These show that an increase in the concentration of NO present in the surrounding gas during irradiation *decreases* radiation sensitivity. The figure shows also that confidence to less than the 0.1 per cent level can be put on the difference between the two slopes that differ only by about 8 per cent. Because of this, we put great certainty on the differences shown in the later figures.

We shall intercompare the effect on radiation sensitivity of oxygen and of NO in different concentrations, during and after irradiation. Figure 2 is a summary statement of the results obtained at 25°C. When the $O_2$ tension in the gas sur-

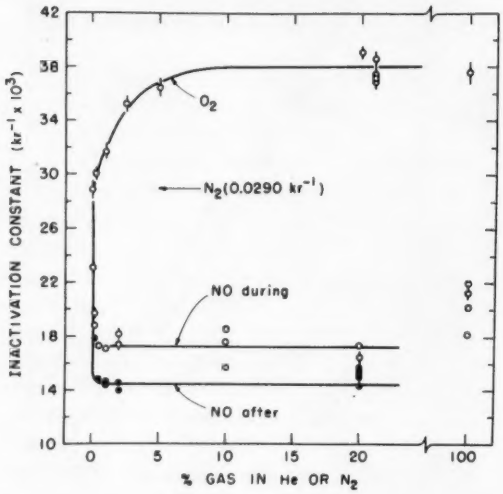


FIG. 2.—The relationship between radiation sensitivity of dry spores and varying concentrations of oxygen during irradiation and NO during and after irradiation before exposure to oxygen. The points are slopes, with standard errors in some instances.

rounding the spores during irradiation is increased from zero concentration, there is a gradual rise in radiation sensitivity to a maximum at about 10 per cent $O_2$ of 1.31 times the $O_2$-free sensitivity. This relationship is the same in nature as those described for bacteria[9] and for ascites cells,[3] but markedly different in degree. In the terms used previously, $m$, the ratio of sensitivities in the aerobic and anaerobic environments is 1.31, which is considerably less than the value of about 3.0 observed in many organisms (see Gray[10] for review), and the oxygen constant $K$ (the concentration of oxygen that brings the response to a value midway between the extremes) is 1.6 per cent.

When the irradiations are performed in the presence of NO of varying concentrations, a similar relationship is observed but its direction is different. Radiation sensitivity drops rapidly with increasing concentration to a saturated value that is 0.60 of that seen after irradiation in $N_2$ followed by exposure to oxygen. The half-value ($K$) is 0.065 per cent NO.

When X-rays are delivered to the spores in $N_2$, and NO is introduced after irradiation but prior to exposure to oxygen, the value obtained is below that seen when NO is present during irradiation. In this instance the slope is 0.50 of that obtained when the spores irradiated in $N_2$ are not exposed to NO; the half value is 0.065 per cent NO. The ratio of these minimal sensitivities (irradiated in the presence of NO on one hand and exposed to NO after irradiation but prior to oxygen exposure on the other) is 1.20, a value close to the ratio of aerobic and anaerobic sensitivities. In this respect oxygen and nitric oxide have similar effects on the radiation sensitivity of dry spores, even though the net effect of NO is a protective one. This must mean that NO has two actions in this dry biological system.

The action of $O_2$ in preventing the postradiation protective action of NO is very significant. As in the case of thermal restoration,[11] brief exposures of the spores to oxygen in the interval between irradiation and the postirradiation treatment always

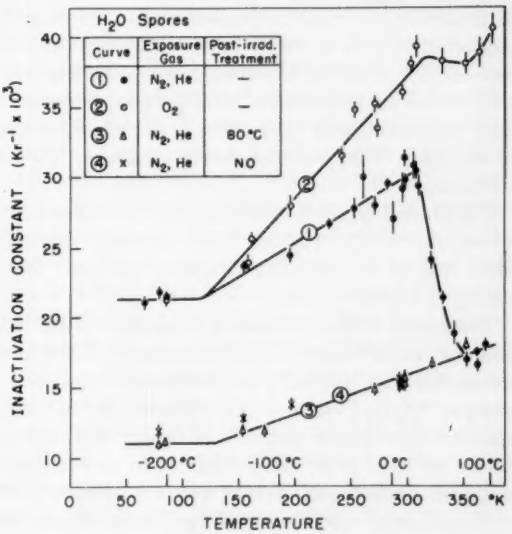


FIG. 3.—A representation of radiation sensitivity as it varies with temperature in certain circumstances. The points "X" are the slopes observed when the spores are treated with NO after irradiation at the indicated temperature but prior to their exposure to oxygen. The slopes observed after heat treatment are taken from ref. 10; the remaining points are from ref. 8.

fixes the radiation damage. These results suggest a correspondence between heat annealment reported by us elsewhere[11, 12] and NO treatment. A series of X-ray exposures in $N_2$ at different temperatures followed by exposure to 3 per cent NO at room temperature prior to exposure to $O_2$ fully confirmed this notion. In Figure 3 the inactivation constants obtained when the spores are exposed to 3 per cent NO after irradiation at various temperatures show a close correspondence to the constants obtained when the spores are heated at 80°C for 15 min following irradiation. A restoration of approximately 50 per cent of the radiation-induced damage (or potential damage) is obtained over a broad temperature range for both heat and NO post-treatments. Furthermore, successive postirradiation treatments of heat and NO do not have additional effect. Thus, the protective action of heat and NO appear to involve the same radiation-induced states in the dry spore.

Two additional temperature relationships of significance are demonstrated by Figure 3. The first, an increase in sensitivity as the temperature is increased, is preserved even after the reversal by heat or NO of about half the damage induced by the radiation. The second, a change from independence of X-ray sensitivity to temperature below about 125°K to dependence above that temperature, is preserved after restoration with heat and is consistent with the constants obtained after restoration with NO. The processes responsible for this relationship to temperature appear to be different from those that can be modified by these postradiation treatments. In other words, changes in temperature affect processes or states that seem to be immediately determined and are independent of and different from the longer-lived states that can be reversed by NO and heat.

In addition to the thermal annealing process that takes place in the absence of oxygen, another oxygen-independent process takes place at a relatively slow rate at usual temperatures. As in the case of the heat experiments,[11] there can be demonstrated in these NO experiments a slow but regular, and apparently oxygen-independent, loss in reversibility on storage of the irradiated cells. This loss in restorability must be interpreted as $O_2$-free fixation of the damaging component in an irreversible, toxic state. Our data for NO and those for the heat treatment are fully consistent with each other. Therefore, in this respect, as well as in respect to the other characteristics described above, NO reversal and thermal annealment are alike.

The $O_2$-free fixation of damage is a heat-dependent process that has an apparent activation energy of about 9,000 calories; this reactivity is appreciably different from that of the thermal annealing process[11] which has an apparent activation energy of 16,000 calories.

*Discussion.*—The convincing evidence[1-4] that NO has the effect of oxygen in enhancing the efficiency of X-irradiation on living cells is directly opposite to our observations that NO has a net protective influence. Examination of the characteristics of NO has led us to an explanation that may eventually reconcile the two apparently contradictory sets of results and, taken with the results reported elsewhere on heat protection, suggests to us certain characteristics of the radiation response in the dry spore that were not apparent previous to these experiments.

Nitric oxide is similar to oxygen in its electronic configuration; it has one unpaired electron and oxygen has two in its outer orbital. This is the reason for expecting[1] that NO would behave like oxygen, with the specific suggestion that oxygen and NO accomplish the same effect on radiation sensitivity because of the reaction of these two substances with radiation-induced species in the cell.

On the other hand, it has been known for many years that NO is a powerful radical scavenger and it has been used routinely to detect the presence of radicals in chemical systems. In these studies, there is a reduction in the concentration of radicals after the introduction of NO. Because of its single unpaired electron, NO in reacting with a free radical should form a complex that is unreactive; the reaction product terminates the reactivity. On the other hand, oxygen, being a biradical, should in combination with a radical produce an oxyradical that is very reactive. On these bases, one should not expect a similarity in action between NO and oxygen.

Therefore, if the radiation effect or part of the radiation effect is brought about

by free radical intermediates which can react with NO, one can expect a protective action of NO in the biological system; on the other hand, oxygen in reacting with these same radicals might not be expected to produce a substance which is inert or harmless, since in simple combination with a radical it should not yield a nonradical species.

Our results in the dry spores are consistent with these considerations. When the spore is exposed to oxygen after irradiation in nitrogen but previous to exposure to NO, the postradiation protective action of NO is prevented. With the methods we use in handling the gases, NO is not seen to detoxify the compounds formed by the reaction of oxygen with the radiation-induced intermediates. If, however, NO is allowed to react with these intermediates before the introduction of oxygen, they are not toxic to the cell. We conclude, therefore, that X-rays form free radicals which have sensible lifetimes in this almost dry cell, and these when combined with oxygen are lethal to the cell. If they can be removed by NO, fully 50 per cent of the radiation effect can be prevented. The effect of thermal treatment after irradiation[11] being the same biologically must be due to the same cause—the removal of free radicals by annealment. We interpret the results of Kempton and Maxwell[13] in dry seeds of *Zea* in the same way—thermal annealment at elevated temperatures results in lowered radiation damage because of the removal of free radicals that would become toxic if oxygen were allowed to react with them. Although in these experiments the seeds were exposed in air, the embryos were probably anoxic because the results paralleled the anoxic spore results. Anoxia is probably brought about in the seeds by endogenous respiration coupled with the relative impermeability of the seed coat (anoxia in seeds exposed to air has been proposed by Gray[10] to explain other radiobiological results). The oxygen is admitted to this system at the time of hydration.[14] In this way the results reported by Kempton and Maxwell,[13] Caldecott[14] and Konzak *et al.*[15] are made consistent with the spore results. Certain elements of this general theory of the oxygen effect are used also by Sparrman *et al.*[5] to explain their results.

The chemical processes following energy absorption are of two kinds: those independent of $O_2$, and those involving oxygen. The kinetic studies we have been able to do thus far indicate that there are two fates possible for the radicals produced by radiation when they are held after irradiation with no exposure to oxygen or a scavenging agent. The gradual loss of restorability with time seen in both the heat[11] and NO studies indicates a slow fixation of the radicals in a lethal state; and the gradual drop in the inactivation constant that can be demonstrated by holding the spores for periods of time prior to exposure to oxygen indicates a slow removal of the radicals in some way that is harmless to the spore. The first of these can be considered a consequence of the migration of energy about the spore to sites that are important for the continued existence of the spore; and the second as the slow migration of energy to traps that are not important for the continued existence of the spore. The exact measurements of these kinetics will be a powerful aid to the understanding of what the energy is, and how it moves about.

The oxygen-dependent processes may involve reactions such as

$$R^{\cdot} + O_2 \rightarrow RO_2^{\cdot} \qquad (1)$$

in which an oxyradical complex is formed. This would result in the presence of a

powerful oxidizing agent in the cell. Support for this type of reaction is given by the EPR studies[16] that demonstrate the growth of an oxyradical signal from doublet and triplet signals when spores, irradiated anaerobically, are exposed to oxygen. A reaction that gives similar biological expectations is

$$O_2 + e^- \rightarrow O_2^- \qquad (2)$$

in which oxygen in the spore collects an electron resulting in the formation of the $O_2^-$ ion—an oxidizing agent more powerful than $O_2$. In either reaction type (1) or (2), we can say that $O_2$ is activated from a not-so-active into a highly reactive species that can secondarily react with important molecules in the spore. This principle—the activation of oxygen into a highly reactive state—is invoked as an explanation of the action of oxygen in certain studies in radiation chemistry by Haissinsky,[17] and is discussed by Gerschman[18] with reference to biological effects of X-radiation.

The high reactivity of the activated oxygen or the oxygen complex can explain our failure to observe an effect of NO on the spores exposed to oxygen after irradiation. Because of the necessity of pumping off $O_2$-containing gas before the introduction of NO in order to avoid reaction between the two gases, an appreciable length of time (minutes) intervenes between oxygen exposure and NO exposure. This is adequate time for disappearance of even moderately reactive compounds and probably sufficient for the fixation of the damage in the spore. Techniques for more rapid changes in the gas environments must be developed to study the characteristics of the oxyradicals that are important for the biological effect.

Several investigators have discussed the question of the role of oxygen in biological systems damaged by irradiation (the most comprehensive recent survey is that of Gray[10]). The "metionic" theory of Alper[19, 20] includes the notion of the reaction between oxygen and damaged "targets," with the reaction being reserved to the "target." Howard-Flanders and Moore[21] propose that radicals are formed, that they revert to harmless states in the absence of $O_2$ and that with either NO or $O_2$ they become harmful complexes. These authors take no position concerning the "target" nature of the radicals.

Our experimental demonstration proves that oxygen plus the radiation-induced radicals adds to the radiation injury observed in the irradiation spore, but it does not require that the "target" is involved. Indeed, the evidence from the EPR studies[16] on the slow development of the finally realized signals indicates that the primary events are not necessarily the finally important ones. With regard to the Howard-Flanders and Moore suggestion,[21] our interpretation of the oxygen-radical relationship does not include a similar action for NO. In fact, according to our interpretation, this is not to be expected.

In Alexander's[22] discussion of the oxygen effect in dry trypsin, the two general possibilities mentioned here—the formation of a radical that secondarily reacts with $O_2$ to induce damage, or the formation of reactive $O_2^-$ by electron capture—are examined with relation to the 50 per cent oxygen effect that is observed when oxygen is present during irradiation of the enzyme. We believe that the explanation is being applied to the incorrect portion of the oxygen effect. As we have pointed out in another communication[12] the general radiation effect in the dry spore system is divisible into three classes of damage. Class I is that which appears

to be the most nearly immediate, and is independent of $O_2$ either during or after irradiation. Class II is that damage which is observed only if $O_2$ is present during the time of irradiation. And Class III is that which is accomplished if the radicals produced by radiation either react with $O_2$ or in some other way activate $O_2$ into a reactive state. This interaction with oxygen may take place some time after irradiation. We believe that activation of oxygen accounts for that part of the radiation effect which is included in the Class III damage. In the case of trypsin[22] this concept is being applied, and incorrectly, we think, to that portion of the damage that we call Class II, that is, the damage that occurs only if oxygen is present at the time of irradiation.

The explanation being entertained at the present time by us for the immediate oxygen effect (Class II) in dry spores, and, as it may turn out, for other dry systems such as enzymes, is different from the one being applied to the Class III damage. The action of oxygen might be the same as that of NO, since they appear to accomplish the same effect. Reaction with radicals is not an altogether satisfactory explanation since, as we have pointed out above, a reactive product is produced in the case of $O_2$ and one that is probably unreactive in the case of NO. However, radical reaction with either $O_2$ or NO could result in damage if the radical involved is an important "target," for effective destruction of the target can conceivably follow its reaction with either.

Oxygen and NO have one action in common that is amenable to test with reference to the immediate effect, namely, they act as quenchers of fluorescence and phosphorescence. This action we conceive of as the conservation of the energy within the spore that would have been liberated as photons from molecules excited by radiation, and its subsequent utilization in the cell to bring about some toxic action. This photon emission should take place very soon after excitation at the high temperatures at which the effect is being tested, and should then be prevented only if oxygen and NO are present *at the time of irradiation*, and brings about Class II damage. At this time we have no ideas concerning the relationship of the details of this process to the inactivation process.

The exact reconciliation of these results with the observations reported for wet, metabolizing systems for the action of NO remains a problem. In the explanation we offer for our results, NO acts like $O_2$ in enhancing the effectiveness of X-rays for a small portion of the total enhancing effect only. The "quenching" proposal may apply to the wet system also, but, in this case, its magnitude is markedly different (300 per cent in contrast to our 125 per cent). In fact, this may be the reason for the similar actions of $O_2$ and NO in the photodynamic breakage of chromosomes.[23]

If, on the other hand, the quenching (or similar) action operates to the same extent in the wet and dry systems, then an additional explanation of the action of NO must be sought for the wet cell. We think that enhancing effects of NO in the wet system that are different from the action of oxygen must be considered seriously and attempts should be made to exclude them experimentally. For instance, if NO forms reaction products with irradiated water that act synergistically with X-rays on the cells, this action cannot be equated to the action of oxygen. In this respect the low-grade toxicity observed for NO in wet cells in the absence of X-irradiation is suggestive.[1] In any case the existence of radiation-induced radicals in the wet

cell, their lifetimes, their reaction with $O_2$ and NO, and the consequences of these remain open questions, although, of course, several explanations can be proposed. The information we present for the dry spore forms at this time a basis for the methodical inquiry into the role of moisture in these effects. If experiments with the seeds of higher plants[5, 13–15] are designed to isolate the various components of the over-all response, they should yield valuable information in this area.

*Summary.*—The radiation sensitivity of dry spores of *Bacillus megaterium* is influenced by the presence of the gases oxygen and nitric oxide both during and after irradiation. When irradiated in the presence of oxygen, the spores demonstrate maximal sensitivity. A reduction of about 30 per cent in sensitivity is observed when irradiation in nitrogen is followed immediately by exposure to oxygen. The radiation sensitivity observed in nitrogen can be reduced one-half by post-irradiation exposure to nitric oxide prior to exposure to oxygen. These results are interpreted to indicate the formation by radiation of long-lived free radicals which in combination with oxygen form irreversible lethal substances (referred to formerly as Class III damage). The action of nitric oxide is the harmless removal of these radicals. However, when irradiation is performed in the presence of NO, the radiation sensitivity is approximately 20 per cent higher than that seen when NO is given after irradiation. This, having about the same magnitude as the immediate oxygen effect, is equated to it, and we propose that this damage (Class II) is brought about by an action of the two gases that is similar. Such an action is the quenching of luminescence that conserves energy within the spore with a corresponding increase in damage. This action of $O_2$ and NO is to be distinguished from the postirradiation action of oxygen which is interpreted to be the result of the activation of oxygen in the cell by the products of radiation.

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## *MOLECULAR REORIENTATION AS UNIFYING PRINCIPLE UNDERLYING CELLULAR SELECTIVITY*

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Specificity is a most basic, though poorly understood, attribute of living systems. It is manifested in the selectivity with which a cell accepts or rejects interaction with outer agents—molecules, particles, or other cells—in accordance with whether or not they are fitting. It rules in such diverse phenomena as food uptake, drug action, hormone response, immune reaction, virus infection, fertilization, cell aggregation, and impulse transmission; all involving a feature of *discrimination* in the determination of whether or not a given effect is to take place.

Ehrlich was presumably the first to ascribe the discriminatory mechanism to the presence or absence of configurational correspondence (steric conformance) between molecules of the agent and of the cell. He visualized the molecular matching as that between a lock and key. This key-lock concept has gained favor ever since. Pauling[1] gave it stereo-chemical concreteness for immune reactions. Lillie,[2] and later Tyler,[3] applied it to the matching of egg and sperm in fertilization, and I,[4] to the selectivity among neurons and other cell types in development and wound healing; and these are just examples. They all point to a common denominator in terms of interactions between molecules of complementary configuration.[5]

The diagrams in Figure 1 symbolize this concept. Steric conformance permits intimacy of approach, hence, interaction; while nonconformant combinations remain too far apart to interact. Yet, in this static form, the concept appears incomplete. One of the complications is that the discriminatory responses referred to often entail a major flux of substance, particles, or currents into or from the affected cell. Such hydrodynamic or electric flux is powerful, but too crude and elemental to be credited with the fine degrees of specificity required to account for selectivity. Conversely, the subtle key-lock interactions of the border molecules which determine the specificity of the effect need not at the same time have to be credited with the force necessary for the massive effects that ensue, particularly if one bears in mind that in many instances only a small fraction of the cell surface is engaged, as in fertilization, agglutination, phagocytosis, or the affine reaction between cells in temporary contact.

In an attempt to account for this dualism, I am proposing in the following a major supplement to the key-lock model, already tentatively outlined in an earlier paper.[6] Logically, it is implied in the key-lock concept. For the function of a key

is not just to fit geometrically into a lock and remain static, but to unlock the door for passage; and what then passes through, is wholly unrelated to the key. The key is merely a means of opening passage for something else. Similarly, an interaction bearing the stamp of specificity may have to be viewed as composed of two steps of different character—a primary initiating key step, which carries specificity, and a secondary implementing step, devoid of specificity, but massive in its effects.

It turns out that such a view finds reasonable support if one considers more closely and concretely the molecular fabric of the cell surface, which evidently is the site at which an agent approaching the cell is subject to its first screening test. By all accounts, the cell surface is a mosaic of lipids and proteins of varying com-

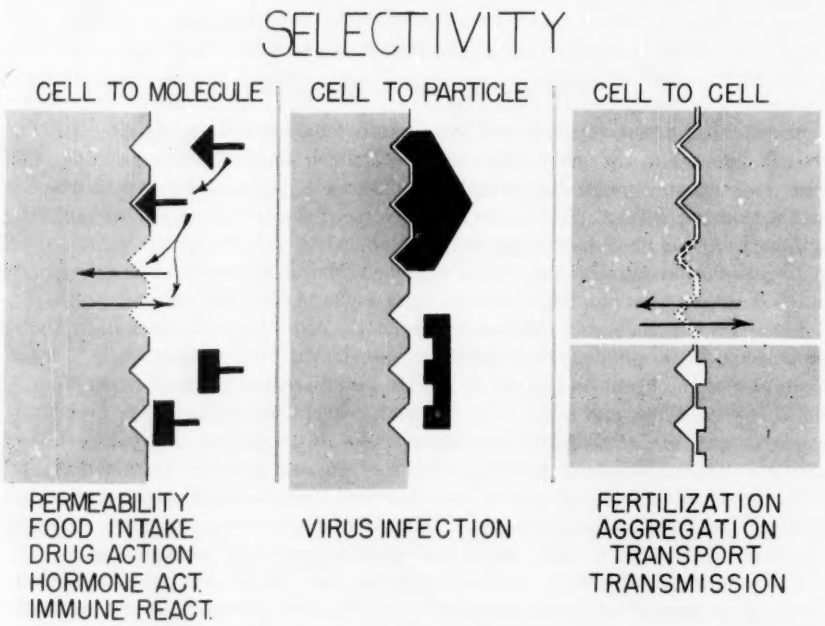


FIG. 1.—Diagrammatic representation of selective acceptance (top portion) or nonacceptance (bottom portion) by a cell surface of molecules, particles, or cells of conforming (top) or nonconforming (bottom) configurations, respectively. Arrows indicate the resulting flux across the cell boundary.

position, constitution, and grain size.[7] The crucial point in the present context is that a major portion of this molecular population is composed of long filamentous protein chains. Now, the longer in relation to width anisodiametric molecules are, the greater becomes the importance of their dimensions and orientations in determining their array in space, or what might be called the texture of the macromolecular fabric. As for the long protein chains present in the cell surface, we must visualize them as lying in the plane of the surface, loosely interconnected into a cohesive network.

Let us assume now that these protein macromolecules possess end groups of highly specific configuration, capable of functioning as acceptors for molecules of com-

plementary configuration. Let us then contemplate what will happen when a unit carrying such complementary compounds approaches a given point of the cell surface.

As is indicated in Figure 2, we may expect the specific end groups of the corresponding surface molecules to become strongly bonded to their complements. Because the latter impinge on the cell surface in essentially radial direction, the backbones of the surface molecules will thereby be forced to rise from their prone into upright positions—a formerly tangential orientation giving way to a preferentially radial alignment.

In the case of short molecules, such reorientation would alter the surface coverage very little. But for long molecules, it implies that a large part of formerly covered surface area is suddenly uncovered. In other words, an erstwhile tight covering

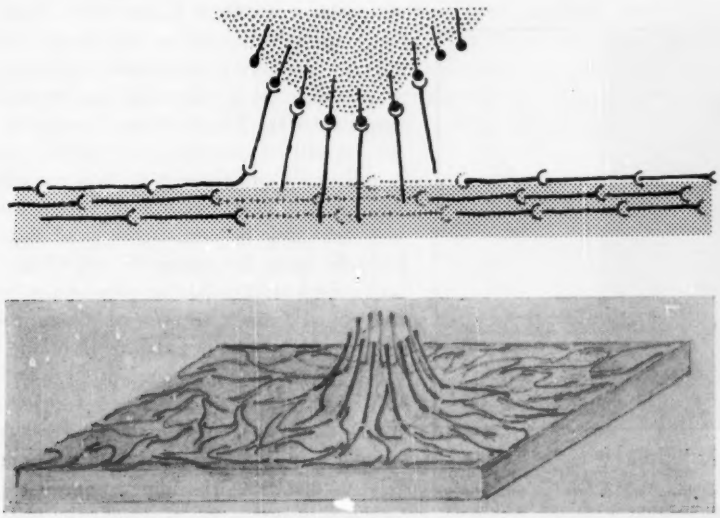

FIG. 2.—Diagrammatic representation of the radial reorientation of tangential surface molecules with specific end groups in the vicinity of an external carrier of complementary groups, resulting in a local "microbreach" of the cell boundary.

springs leaks, and such breaches obviously form temporary channels for substance traffic on a larger scale. If we view the tangential fibrous coat of the resting surface as a barrier to such cross traffic, the righting at an excited site of molecules into radial positions amounts to the opening of a gate through the barrier. I shall, therefore, speak figuratively of "barrier" and "open-gate" positions.

The magnitude of the effect is visually expressed in Figure 3. The left half shows 42 lines symbolizing 42 linear molecules with an axial ratio (of length to width) of 100; this corresponds approximately to the shape and dimensions of collagen molecules. The packing in this diagram is assumed to cover the surface pretty tightly. The right half shows the same 42 molecules in scale after they have been turned perpendicular to the surface, so that only their cross sections partake of the surface. Since at an axial ratio of 100, the cross sections occupy only one per

cent of the formerly covered area, 99 per cent of that area has become denuded as a result of the reorientation. Evidently, the contrast shown in the diagram represents an extreme condition, for in reality only a fraction of surface molecules will be affected, and even those that are may be lifted out of the surface only for part of their lengths.

Besides yielding gaps in the barrier, radial reorientation may, however, also have a positively expediting effect on cross traffic, as follows: If the radially realigned molecular chains were to aggregate into filaments, these would constitute straight bridges between two units of an interacting pair, so as to serve as channels for oriented transport of substance, or even perhaps conduction of currents, if a comparison to semiconductors is not too farfetched.

Some consideration must also be given to the probability that oriented streaming of substances and currents across such breaches would serve to accentuate the molecular orientation in the radial direction of the stream lines. Since this would hold not only for the border molecules, which were primarily affected, but equally for the molecules swept into the breach from the inside or outside, it will be seen that an originally tenuous connection could thereby become consolidated. Although initiated by a specific interaction, any leak may thus be perpetuated and kept open for some time by unspecific sequelae. These latter may involve lasting attachment between the joined masses, transfer of substances from one into the other, or engulfment of one by the other. The "healing" time of an open breach will vary according to circumstances, as the free ends of the molecular fringe will tend to become reunited at rates depending upon their relative distances, the ionic composition of the medium, cohesive forces within the cell, etc.

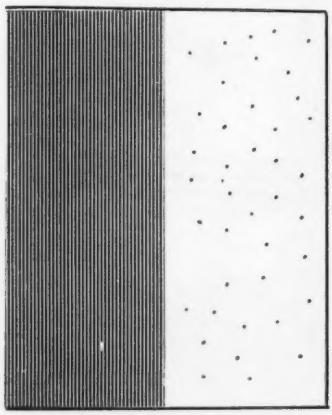

FIG. 3.—Change in the coverage of a given surface area resulting from the turning of a given number of filaments from positions within the surface (left) to positions normal to the surface (right).

Next to the selective gate-opening by key compounds, we must envisage the possibility that point sources of electric discharges might produce the same effect by less specific means. Figure 4 illustrates how a dense cloud of electrons pointed at an electric double layer, such as is present in the surface of a cell or of a nerve fiber, may not only cause local depolarization but, if it is renewed, force filamentous surface molecules again from barrier into open-gate positions, with secondary effects fully comparable to those following more specific initiations.

This possibility is particularly intriguing in view of its possible pertinence to excitation in the nervous system. Electric stimulation of the peripheral nerve fiber, as is well known, is effective only if the charge density at a given point reaches a certain threshold minimum. Peripheral nerve fibers of the myelinated type are excitable chiefly at the nodes. Might it not be that the electric stimulus exerts its primary effect by the assumed reorientation of longitudinal molecules in the axonal surface into radial positions, thus opening pores for the secondary passage of ions

and their carrier molecules. The functional significance of the myelin coat would be to prevent the springing of such leaks in the internodes and hence avert the spreading of excitation to adjacent fibers.

Impulse transmission from one unit to another in the nervous system is still another matter. Undoubtedly, electric polarization and depolarization and the liberation and inactivation of transmitter substances, such as acetylcholine or adrenalin, are involved, though the manner of their participation is still debated.[8] But there is more that is involved. There is compelling experimental evidence[9] to show that (*a*) the many units composing the neuronal population are not all of one kind, but, on the contrary, belong to a great number of biochemically distinct varieties, and (*b*) that only units of the same or a related variety will enter transmissive relations with one another. The principle of selectivity in intercell relations thus pervades the whole central nervous system and its receptors and effectors. This leads one to suspect that if the cells to either side of a synapse were to contain surface molecules of reciprocally matching character, these molecules would mutually elevate each other from their resting barrier positions into open-gate positions, thereby opening pathways for the transfer of transmitters or the direct passage of current. Conceding, furthermore, to neurons some modifiability of state, one could

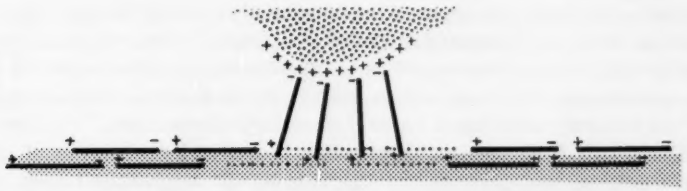

FIG. 4.—Diagrammatic representation of surface breaches produced by local eleletric stimulation.

expect transmission between two morphologically connected units to be open at certain times, but blocked at others. In the transmission from nerve to muscle, perhaps the combination of acetylcholine with an acceptor in the motor end plate serves as the key-lock mechanism for opening the gate for the electric impulse.[10]

These comments mark the first application of our concept to a specific problem. As one can see, it readily resolves the conflict between two apparently inconsistent experiences gained in the study of the nervous system, namely, that between the demonstrably high degree of specificity among the units on the one hand, and the almost total absence of signs of this selectivity in their electric and metabolic activities, on the other.

Many other instances of specific interactions can likewise be accounted for by the outlined dualistic principle. For example, if Tyler[3] assumes the sperm head to be attached to the egg surface by complementary molecular groups, antigen–antibody fashion, while others view the penetration of the sperm as a more complex process, both views can be reconciled by assuming that specificity is inherent in the very first step of the process only—the coupling of matching pairs of surface molecules of sperm and egg—but that the resulting breach in the egg surface opens the way for a chain of secondary events of lesser specificity.[11] In fact, the observation

of a minute cone protruding from the egg and tapering into a filament toward the approaching sperm head[12] is decidedly the kind of morphological expression to be expected from our theory.

Virus infection likewise reveals the dualism of primary selective attachment and secondary more massive transfer. The protein coat of a bacteriophage carries the specific identification marks that make the virus latch on to an appropriate bacterial surface. If this specific combination were again to do no more than create a local surface breach, the subsequent injection of the nucleic acid content of the phage particle into the bacterium would follow as an unspecific consequence. This would also explain why, having served as mediator, the protein coat stays on the outside.[13]

A possible extension of the principle to the uptake of particles in general remains to be explored. Actually, the whole idea of the crucial role of molecular orientation in the cell surface came to me originally from observations on phagocytosis.[14] Certain spindle cell types could be made to flatten to disk-shape on impact with a smooth substratum. During this transformation, silver impregnation revealed fibrous striations, which in the spindly portion of the cell ran parallel to the surface, but in the flattened portion were oriented radially to the margin. Since carmine granules added to the medium had penetrated the living cell promptly in sectors with radial orientation, but never across tangentially oriented barriers, the concept of molecular chinks as channels of entry suggested itself. However, recent electronmicroscopic intimations[15] that small particles are being engulfed in a recess, rather than in a protrusion, of the cell surface, might call this interpretation into question, unless the infolding were again to be a secondary phenomenon. On the whole, the possibility that our concept may have a bearing on mechanisms of selective cellular ingestion, and perhaps even permeability,[13] is presented here only on account of the fact that since we are still lacking a fully consistent general theory of those processes, any plausible hypothesis should at least be examined.

Considerations analogous to those given in the foregoing to the selective intake of materials by the cell from its environment would, of course, apply to traffic in the reverse direction, i.e., the selective extrusion of products from cells. Moreover, the principle would hold as well for the exchange between cytoplasm and cell organelles and between cytoplasm and nucleus. For the latter case, it would resolve the controversy as to whether the "annuli" in the nuclear membrane seen in electronmicrographs of fixed preparations are open pores or are closed, for "pores" in our version may be facultatively either open or closed. All interfaces and membranes of and within cells should be viewed as metastable structures, neither permanently closed nor permanently perforated. Pores of varying sizes would form in response to the proper kind of key molecules, or even potent electrical disturbances, and they would close again upon the subsidence of the local stimulus.[16]

Another application of our hypothesis pertains to the so-called "inductive" interactions between contiguous cells in development. Cellular differentiation occurs in a long chain of separate modifying steps, some of which necessitate the intervention of other cells, often through intimate contact.[6] Such contact interactions have been presented alternatively as involving either molecular reorientation or the transfer of substance. But both alternatives can now be readily combined by letting specific interaction between adjacent cells merely produce the

surface breaches across which massive transfer of substance can then proceed.

Hormone action on endocrine target cells could be interpreted similarly. By reorienting molecular groupings in the cell surface, the hormone would simply render the receptor cell permeable to less specific uptake from, and output into, the cellular environment, with the specificity of the effect again residing solely in the first member of the chain.

Perhaps the relative roles of antibody and complement in lytic immune reactions could likewise find clarification in the light of such a dualistic concept.

The list of examples I have cited is by no means exhaustive. Only experience will tell whether it is too inclusive. At any rate, I am fully conscious of the fragmentary and tentative nature of the whole concept. Yet regardless of the details, its basic tenet can hardly be doubted; viz., that in dealing with macromolecules, a mere recording of their presence without specifying their orientation gives no clue to their functional effectiveness, as reorientation by 90 degrees can transform a barrier into a traffic lane, and vice versa.

*Summary.*—A general hypothesis of the mechanism of selectivity of cellular responses is proposed to embrace a wide spectrum of specific interactions, including immunology, virus infection, fertilization, ingestion, hormone action, cell aggregation, and impulse transmission. It is based on the following assumptions: (1) The cell surface contains a network of long protein molecules with specific end groups. (2) This network acts as "barrier" to transport and transmission. (3) Complementary groups on an extraneous carrier combine specifically with their counterparts in the cell surface and thereby turn them from tangential into radial positions. (4) This reorientation opens wide "breaches" through the former barrier. (5) These breaches then act secondarily as pores or portals for the more massive, but unspecific, passage of materials and currents.

[1] Pauling, Linus, "Molecular Structure and Intermolecular Forces," in *The Specificity of Serological Reactions*, ed. K. Landsteiner (Harvard Univ. Press, 1946).

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[3] Tyler, A., "Gametogenesis, Fertilization and Parthenogenesis," in *Analysis of Development*, ed. B. H. Willier, P. A. Weiss, and V. Hamburger (Philadelphia & London, W. B. Saunders Co., 1955).

[4] Weiss, Paul, *Yale Jour. Biol. & Med.*, **19**, 235–278 (1947); "Specificity in Growth Control," in *Biological Specificity & Growth*, ed. E. G. Butler (Princeton University Press, 1955), pp. 195–206.

[5] See also Burnet, F. M., *Enzyme, Antigen and Virus* (Cambridge Univ. Press, 1956).

[6] Weiss, Paul, *International Review of Cytology*, **7**, 391–423 (1958).

[7] Danielli, J. F., "The Cell Surface and Cell Physiology," in: *Cytology and Cell Physiology*, ed. Bourne (London: Oxford Univ. Press, 1942).

[8] See Nachmansohn, D., *Chemical and Molecular Basis of Nerve Activity* (New York: Academic Press, 1959); Eccles, J. C., "Neuron Physiology—introduction," in *Handbook of Physiology* (Washington: Physiol. Soc., 1959), vol. 1, pp. 59–74.

[9] Reviewed in Weiss, Paul, *Sympos. Soc. Exptl. Biol.*, **4**, 92–111 (1950); Sperry, R. W., *Growth Symposia*, **10**, 63–87 (1951).

[10] It is interesting to consider the fact that we are faced here with the possibility of pairwise reciprocal action mechanisms. On the one hand, a specific interaction can establish a channel for an electric current, but conversely, an electric effect (as in Fig. 4) can prepare a pathway for substance transfer. The latter view has been advanced by J. C. Eccles (*The Physiology of Nerve Cells*, Baltimore: Johns Hopkins Press, 1957). The possibility of both effects being real and appearing in combination cannot be discounted.

[11] See also Tyler, A., "The Cytochemistry of Enzymes and Antigens," *Exper. Cell Res.*, Suppl. 7, 183–199 (1957).

[12] Colwin, L. H., and A. L., *Biol. Bull.*, **110**, 243–257 (1956).

[13] A recent note from the Department of Bacteriology and Immunology of Harvard Medical School (N. Anand, B. D. Davis, and A. K. Armitage, *Nature*, **185**, 22–24, 1960), based on an analysis of the mode of action of Streptomycin, advances a similar dualistic concept, according to which a primary specific step produces leaks in the bacterial membrane to be followed by a secondary massive infiltration. They state: "A drug that damages the permeability barrier of a cell would promote the entry of other drugs."

[14] Weiss, Paul, *Anat. Rec.*, **88**, 205–221 (1944).

[15] Bennett, H. S., *J. Biophys. Biochem. Cytol.*, **2** (supplement), 99–103 (1956).

[16] This concept of facultatively functional pores seems to provide for a larger measure of specificity and adaptive flexibility than the one proposed by J. F. Danielli (in *Recent Developments in Cell Physiology*, ed. J. A. Kitching, pp. 1–14, New York: Academic Press, 1954), although both may be complementary, rather than mutually exclusive.

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## *HARD SPHERE BOSE GAS: AN EXACT MOMENTUM SPACE FORMULATION**

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*Communicated by H. A. Bethe, May 13, 1960*

In every low density solution that has appeared so far for the ground state energy and low lying excited states of the hard sphere Bose gas, a momentum space formulation of the problem has been essential. The reason for this is that at low densities the wave function has a simple structure in momentum space while it is quite complicated in configuration space. That is to say, the wave function describes a situation in which most of the particles are in the ground state; the relatively small number that are excited are, owing to momentum conservation, predominantly excited in pairs with momenta $\mathbf{k}$ and $-\mathbf{k}$. Moreover, since the most convenient way to handle the Fourier transform of a symmetric function is to introduce the second quantized notation, formulating the problem in momentum space is equivalent to second quantization in momentum space.

On the other hand, while the configuration space Hamiltonian is relatively simple, no one has succeeded in finding the exact momentum space (second quantized) Hamiltonian. Approximations to it have indeed been found, the pseudopotential being the most popular approach, but not the exact Hamiltonian valid for all densities. It is the purpose of this note to exhibit the exact equations.

Let us begin with a few definitions. We have $N$ particles in a cubic box of length $L$ ($L^3 = \Omega$); the wave function satisfies periodic boundary conditions on the walls of the box and in addition $\psi(x_1, \ldots, x_N) = 0$ whenever any pair satisfies $|x_i - x_j| = a$. We further define $R$ to be the full $3N$ dimensional space of the particle coordinates and $W$ to be the $3N$ dimensional subspace of $R$ such that all pairs of coordinates satisfy $|x_i - x_j| \geq a$. Then, *inside* $W$, $\psi$ satisfies

$$\sum_1^N p_i^2 \psi = E\psi \tag{1}$$

and $\psi = 0$ on the common boundary of $R$-$W$ and $W$. The definition of $\psi$ in the region $R$-$W$ is quite arbitrary and of no physical significance; we shall adopt the definition $\psi = 0$ in $R$-$W$. The wave function so defined is finite and continuous in $R$ (although with a discontinuous derivative) and hence can be represented by a Fourier series whose coefficients are

$$\varphi(\mathbf{k}_1, \ldots, \mathbf{k}_N) = \Omega^{-N} \int_R \exp\,[i\sum_1^N \mathbf{k}_i \cdot x_i]\psi(x_1, \ldots, x_N) \prod_1^N d^3x_i. \tag{2}$$

Now multiply both sides of equation (1) by $g = \exp\,[i\Sigma\mathbf{k}_i \cdot x_i]\Omega^{-N}$ and integrate over $W$. On the right hand side we obtain $E\int_W g\psi = E\int_R g\psi = E\varphi$. A typical term on the left hand side is $-\int_W g(\nabla_1^2\psi)$ and may be converted into an integral over $R$ by partial integration, and hence into an expression involving $\varphi$. After some manipulation and judicious use of the boundary conditions we obtain

$$\lim_{\epsilon \to 0+} 4\pi a\Omega^{-1} \sum_{\mathbf{p}} \sum_{<i,j>} \varphi(\mathbf{k}_1, \ldots, \mathbf{k}_i + \mathbf{p}, \ldots, \mathbf{k}_j - \mathbf{p}, \ldots, \mathbf{k}_N) \cos\,(|p|\,(a + \epsilon)) =$$

$$(E - \sum_1^N \mathbf{k}_i^2)\ \varphi\ (\mathbf{k}_1, \ldots, \mathbf{k}_N). \tag{3}$$

The summation over $<i, j>$ means a summation over all possible pairs, and $\sum_{\mathbf{p}}$ means the usual summation over momenta. The derivation of (3) is tedious but elementary. The reason for the limit is that we require the normal derivative of $\psi$ on the boundary of $R$-$W$ taken from inside $W$.

It is now trivial[3] to write equation (3) in the second quantized notation:

$$\lim_{\epsilon \to 0+} H_\epsilon \psi = E\psi \tag{4}$$

where $\psi$ now means the second quantized wave function of $N$ particles and

$$H_\epsilon = \sum_{\mathbf{k}} \alpha_k^* \alpha_k + \frac{4\pi a}{\Omega} \sum_{\mathbf{k},\mathbf{q},\mathbf{p},} \alpha^*_{\mathbf{q}+\mathbf{p}}\, \alpha^*_{\mathbf{k}-\mathbf{p}}\, \alpha_{\mathbf{q}}\, \alpha_{\mathbf{k}} \cos\,(|p|\,(a + \epsilon)) \tag{5}$$

Equation (4) is admittedly unusual. Although $H_\epsilon$ is Hermitian and therefore may be regarded as a Hamiltonian, equation (4) is not an eigenvalue problem in the usual sense. It is of central importance to understand what equation (4) does not mean. It might be supposed that since $H_\epsilon$ is Hermitian one should find its eigenfunctions and eigenvalues (which will depend upon $\epsilon$) and take the limit in the following way: $\lim_{\epsilon \to 0+} H_\epsilon\psi_\epsilon = \lim_{\epsilon \to 0+} E_\epsilon\psi_\epsilon$. This is most emphatically not the case. What equation (4) implies is $\lim_{\epsilon \to 0+} H_\epsilon \{\lim_{\epsilon \to 0+} \psi_\epsilon\} = E \lim_{\epsilon \to 0+} \psi_\epsilon$. $E$ is not equal to $\lim_{\epsilon \to 0+} E_\epsilon$.

Such a limiting process has already been noticed in connection with the pseudopotential,[1, 2] which however suffers from two disadvantages: (*a*) it is correct only for low density; and (*b*) the $H_\epsilon$ is not Hermitian although $\lim_{\epsilon \to 0+} H_\epsilon$ is Hermitian. We are therefore led to believe that an equation such as (4) is the best one can hope for and that, in fact, no momentum space Hamiltonian exists in the usual sense. Two considerations lead us to this view. The first is that if there were a Hamiltonian, its eigenfunctions would form a complete set in $R$. But the original prob-

lem, equation (1), defines a complete set only in $W$. Hence such a Hamiltonian would have too many solutions and would have to be modified by some subsidiary condition. The second reason stems from the observation that for the ground state

$$E = 4\pi N\rho\alpha\left\{1 + \frac{128}{15\sqrt{\pi}}(\rho\alpha^3)^{1/2} + \ldots\right\},$$

the second order term being positive. If $\psi_0$ is the unperturbed ground state function, then $S = \langle\psi_0|H|\psi_0\rangle$ would give an upper bound for $E$. But we would expect that $S = 4\pi N\rho\alpha$ for low density and consequently the second order term would be negative as is the case for a finite potential.

The solution of (4) leads to the well-known expression for the ground state energy given above, provided one makes the Bogoliubov approximation. This approximation has been used by all workers in the field, but the author has serious doubts that it is adequate—at least for the repulsive core. In short, the author feels that the coefficient, $128/15\sqrt{\pi}$, cannot be regarded as definitely established, and intends to discuss the solution of equation (4) as well as the details of its derivation from equation (1) in a subsequent paper.

In conclusion it should be remarked that while we have proved that $\psi$ satisfies equation (4), it need not be true that all solutions to (4) are admissible, i.e., that they correspond to functions in configuration space that vanish in $R$-$W$. This point is not clear, but if it turns out to be necessary to invoke this subsidiary condition it may be expressed in second quantized form in the following way:

$$\lim_{\epsilon \to 0+} \sum_{\mathbf{k},\mathbf{q},\mathbf{p}} \alpha_{\mathbf{q}+\mathbf{p}}^{*}\,\alpha_{\mathbf{k}-\mathbf{p}}^{*}\,\alpha_{\mathbf{k}}\,\alpha_{\mathbf{q}}\,\frac{1}{\rho^2}\left\{\cos(|p|\,(a+\epsilon)-)\,\frac{1}{|p|\,(a+\epsilon)}\right.$$
$$\left.\sin\,(|p|\,(x+\epsilon))\right\}\psi = 0. \quad (6)$$

Equation (6) is a necessary and sufficient condition that $\psi$ vanish in $R$-$W$, but it proved to be completely unnecessary to use it for the evaluation of the first few terms in the energy. In reality, the very fact that $\psi$ satisfies equation (6) proves to be a help in solving equation (4) rather than a hindrance. The additional piece of information supplied by equation (6) actually makes the analysis of the repulsive core problem easier than that of any finite potential. It should also be remarked that equations (1) through (6) are exact for any $N$, even $N = 2$, provided we adhere strictly to the periodic boundary conditions.

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[1] Lee, Huang, and Yang, *Phys. Rev.*, **106**, 1135 (1957).

[2] Wu, T. T., *Phys. Rev.*, **115**, 1390 (1959).

[3] The reader may satisfy himself of the transition from (3) to (4), by finding the analogue of (3) for a finite potential and comparing the result with the well-known second quantized Hamiltonian for a finite potential. $8\pi a\cos(|p|\,(a+\epsilon))$ is simply replaced by $v(\mathbf{p})$, the Fourier transform of the potential, in the finite case.

# *RADIOANGIOGRAPHIC STUDIES OF CIRCULATION IN THE MATERNAL PLACENTA OF THE RHESUS MONKEY: PRELIMINARY REPORT*

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*Communicated by R. K. Burns, May 2, 1960*

It has been the consistent testimony of recent anatomical studies of the maternal placenta of primates that the *vis a tergo* of maternal arterial blood pressure is the controlling factor effecting circulation of blood through the placenta.[1,2]

The primate placenta is of the hemochorial type in which clusters of fetal chorionic villi containing the fetal blood vessels hang free in a pool of maternal blood, the

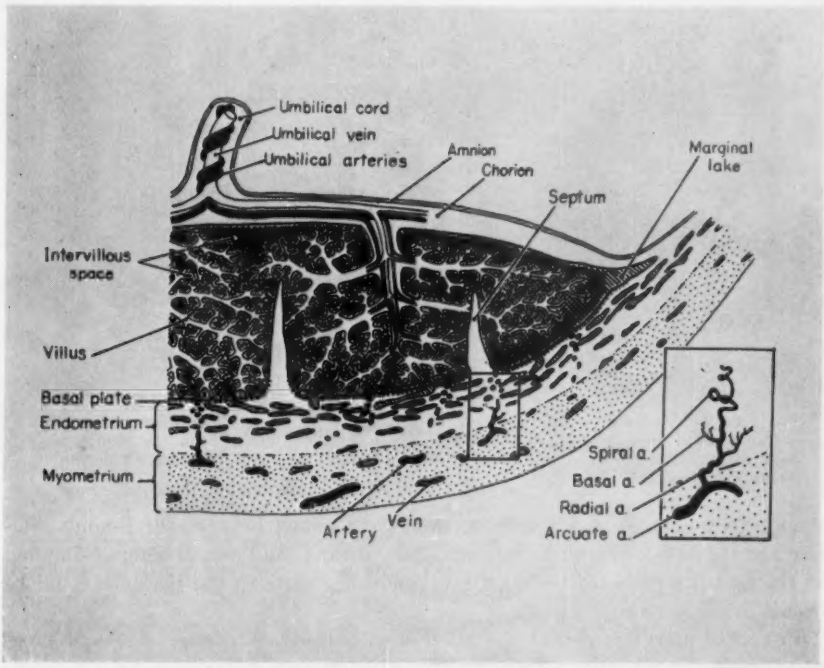


FIG. 1.—Diagrammatic representation of a portion of the primate (hemochorial) placenta attached to the uterine wall. The inset shows, at higher magnification, the components of a single arterial stem. (From Ramsey,[3] in *Report of 15th M & R Pediatric Research Conference* 1955.)

intervillous space. Maternal blood is supplied to this pool through the base of the placenta by endometrial branches of the uterine arteries. Blood is drained from the intervillous space by endometrial branches of the uterine veins (Fig. 1). Exchange of metabolites between mother and fetus takes place across the tissue of the villi in whose fibrous core the fetal blood vessels are embedded.

Experimental procedures employed in the Carnegie Department of Embryology to study the circulation in the primate placenta have been as follows.

In rhesus monkeys studied at close intervals throughout pregnancy one or another of three techniques was used:

1. Intra-aortic injection of India ink at laparotomy with subsequent removal of the uterus and serial sectioning of all or part of the placenta *in situ* and the full thickness of the adjacent uterine wall.[3]

2. Injection of nontoxic colloidal mercuric sulfide into the femoral vein with subsequent laparotomy and removal of the uterus when the dye has been fully distributed through the systemic vascular bed. The placenta was sectioned as in the first technique.[4]

3. An "auto-injection" procedure whereby, at laparotomy, the *in situ* uterus was immersed in copious amounts of Bouin's solution for periods of from 4–7 min. The uterine ligaments and their vessels were then clamped simultaneously and

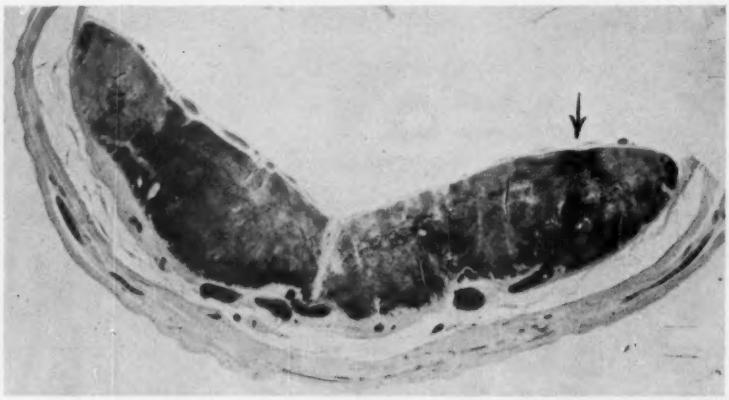

FIG. 2.—Mercuric sulfide injection of a monkey placenta. The arrow indicates the location of a spurt of dye high into the intervillous space from the mouth of an endometrial arteriole. Monkey C-747. 121 days pregnant. Primary placenta. Section B 17. × 2.

the uterus was removed for serial sectioning. Contact between the Bouin's solution and the blood, which was still circulating during the interval before clamping, so changed the color of the blood that its course through the placenta could be traced in the sections.[2]

In human uteri removed at operation or at autopsy at various stages of pregnancy from the 8th week to term, injections of India ink were performed. Canulas were inserted in one or both uterine arteries immediately after removal of the specimen and the vascular bed was flushed at pressures below diastolic arterial pressure with an isotonic saline solution to which 1 per cent sodium nitrate and 0.1 per cent histamine had been added. This perfusion was followed by India ink injection at the same pressure. The placenta was studied by serial sections as in the monkey experiments.[1]

Plastic sheet reconstructions of human and rhesus material were prepared to show the changing character of the uterine vascular bed during pregnancy.

Whenever the point of connection of a maternal artery with the intervillous space was encountered in the sections it was noted that the maternal blood (or injection material) entered the placenta in the form of a "spurt" or fountain-like "jet" which carried the blood in a discrete mass far toward the chorion before lateral dispersion occurred (Figs. 2 and 3). Repeated observation of this phenomenon suggested interpretation of the circulatory mechanism in terms of the following "*physiological concept*":

"Arterial blood enters the placenta from the endometrial arteries under a head of pressure sufficiently higher than that prevailing in the vast, amorphous lake of the intervillous space that the incoming stream is driven high up toward the chorion. Gradually this force is spent and lateral dispersion occurs, aided by the villi which, acting as baffles, promote mixing and slowing and by their own pulsation effect a mild stirring. Eventually the blood in the intervillous space falls back upon the orifices in the basal plate which connect with maternal veins, and

FIG. 3.—Autoinjection of a monkey placenta. Bouin's bath of *in situ* uterus. Maternal blood, colored by contact with Bouin's solution, spurts high into the intervillous space at the point marked by the arrow. Monkey C-750. 123 days pregnant. Secondary placenta. Section 100. × 2.

since there is an additional fall in pressure between the intervillous space and the endometrial veins, drainage is accomplished."[2] This circulatory progress is enhanced by intermittent myometrial contractions throughout pregnancy.

Physiological studies to test this working hypothesis have been carried out in the monkey. Pressure transducer systems have been introduced simultaneously into the femoral artery and femoral vein, the intervillous space of the placenta and the amnionic cavity in early, mid, and late pregnancy. Although pressures in the femoral artery and femoral vein are only roughly representative of the effective arterial and venous pressures within the uterine wall at the placental base, the data obtained have confirmed the pressure gradients assumed in the physiological concept and established the existence, character, and effect of the myometrial contractions.[5]

Highly suggestive as these results appear to be, they must be regarded as indirect evidence only, because of the artificial conditions introduced into the ex-

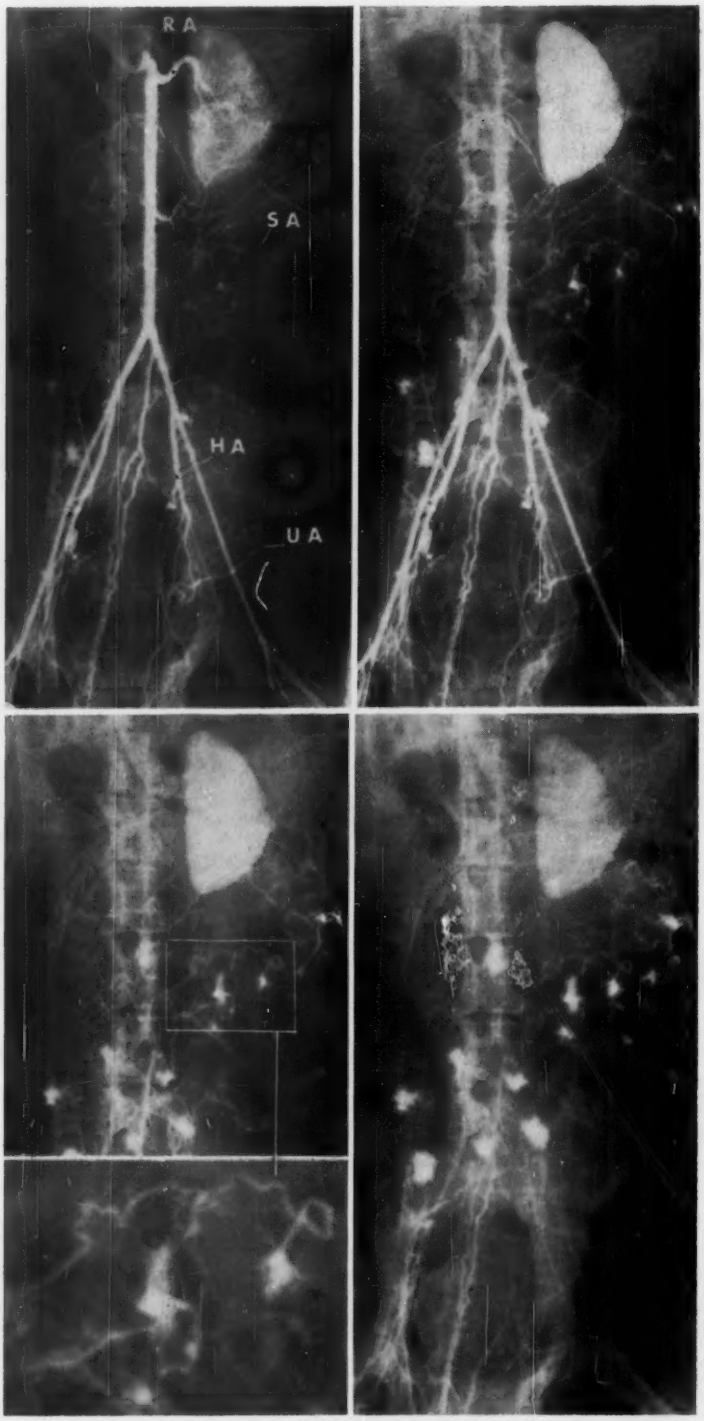


FIG. 4.—Photographs of four of a series of radiograms made at $3^1/_2$, 4, 5, and 6 sec, respectively, following injection of a radiopaque dye into the right femoral artery of Monkey 12B, 111 days pregnant. The insert in the third photograph (lower left) is a magnification (×4) of the area inclosed in the box. The arrows indicate spurts of dye into the intervillous space. RA, renal artery; SA, spiral artery of endometrium; HA, hypogastric artery; UA, uterine artery.

periments. In the search for a more physiological procedure we have gratefully adopted a suggestion of Dr. Louis M. Hellman who proposed that we employ the technique of radioangiography with which he had performed some preliminary studies in the human. This procedure, originally worked out by Fernström[6] and Borell,[7] has limited usefulness for prolonged or repeated investigations in humans because of the associated risk of overexposure to radiation.

The pregnant monkey is anesthetized with intravenous pentobarbital sodium and one femoral artery is exposed under sterile conditions. A 20-gauge needle is then inserted into the artery counter to the flow of blood. The needle is connected, by means of a polyethylene catheter, to an automatic pressure syringe containing 15 cc of a 90 per cent solution of diatrizoate sodium (Hypaque), a well-tolerated tri-iodine, radiopaque dye. Under 90 lbs. of pressure delivered from a cylinder of compressed nitrogen the dye is instantaneously injected into the artery and at the same time rapid serial radiography is commenced. X-ray tubes fixed at right angles to each other permit simultaneous anteroposterior and lateral views to be taken. Exposures of 80 kv, 500 ma at $^1/_{20}$ sec for the AP view and 95 kv for the lateral have been found to be optimum for a 5,000 to 6,000 gm monkey in mid-pregnancy. The X-ray films are contained in an automatic film changer (Schönander) set to deliver two films per second for a total of 13 seconds. Single preliminary and post-experimental films are made to verify the stage of pregnancy and to check the time of clearance of the dye by the kidneys.

The representative X-rays reproduced in Figure 4 illustrate the manner in which the retrograde progression of the dye may be followed from the point of injection. The dye courses up the femoral artery under pressure into the aorta and reaches the level of the left renal artery just as the applied pressure is spent. It then returns under the force of arterial pressure and enters the hypogastric arteries, the uterine arteries and finally the intervillous space. The characteristic spurts appear as the dye enters the placenta.

*Discussion.*—The artificial conditions imposed by barbiturate anesthesia and incision into the femoral triangle do not alter the uterine circulation. Confirmation of this lies in the similarity of the circulatory pattern observed in the monkey with that in the unanesthetized human patients in whom injection was made through the intact skin.[6,7] The high injection pressure is an essential part of the technique in order to avoid dilution and dispersion of the dye before it reaches the uterine arteries. As mentioned above, the excess pressure is dissipated before the dye enters the uterine vessels. Further evidence that the spurts have not been caused artificially lies in the fact that identical formations were obtained in the second type of anatomical study described above in which maternal pressure was the only propulsive force (Fig. 2).

Studies currently in progress are directed toward visualization of the venous drainage of the intervillous space and toward establishment of the circulatory pattern at various stages of pregnancy and under different conditions of myometrial activity.

It is concluded that radioangiography affords a physiological method for study of the circulation within the maternal placenta. Use of this technique in pregnant monkeys has provided results in full harmony with those obtained by anatomical studies and confirmed by pressure recordings. The uniformity of the results

obtained by at least six different methods of study confirms the "physiological concept" that the circulation of blood in the maternal placenta is controlled by the head of pressure in the maternal arterial system.

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**Couch, John Nathaniel**, 1943 (7), University of North Carolina, Chapel Hill, North Carolina

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**Crawford, Bryce, Jr.**, 1956 (5), Graduate School, University of Minnesota, Minneapolis 14, Minnesota

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**Danforth, Charles Haskell**, 1942 (8), Department of Anatomy, Stanford University, Stanford, California

**Daniels, Farrington**, 1947 (5), Department of Chemistry, University of Wisconsin, Madison 6, Wisconsin

**Davidson, Norman** Ralph, 1960 (5), Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena 4, California

**Davis, Hallowell,** 1948 (9), Central Institute for the Deaf, 818 South Kingshighway, St. Louis 10, Missouri

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**Deutsch, Martin,** 1958 (3), Laboratory for Nuclear Science, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

**Dingle, John Holmes,** 1958 (10), School of Medicine, Western Reserve University, Cleveland 6, Ohio

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**Esau, Katherine**, 1957 (7), Department of Botany, University of California, Davis, California

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**Ewing, William Maurice**, 1948 (13), Columbia University, Lamont Geological Observatory, Torrey Cliff, Palisades, New York

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**Fenn, Wallace Osgood**, 1943 (9), School of Medicine and Dentistry, University of Rochester, 260 Crittenden Boulevard, Rochester 20, New York

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**Fisk, James Brown**, 1954 (4), Bell Telephone Laboratories, Incorporated, Murray Hill, New Jersey

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**Gasser, Herbert Spencer**, 1934 (9), Rockefeller Institute, New York 21, New York

**Gates, Marshall** DeMotte, Jr., 1958 (5), Department of Chemistry, University of Rochester, Rochester 20, New York

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**Gilliland, Edwin Richard**, 1948 (4), Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

**Gilluly, James**, 1947 (6), United States Geological Survey, Denver Federal Center, Denver 25, Colorado

**Gilman, Henry**, 1945 (5), Department of Chemistry, Iowa State University, Ames, Iowa

**Glass, Hiram Bentley,** 1959 (8), Department of Biology, Johns Hopkins University, Baltimore 18, Maryland

**Goddard, David Rockwell,** 1950 (7), Division of Biology, University of Pennsylvania, Philadelphia 4, Pennsylvania

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**Graham, Clarence Henry,** 1946 (12), Department of Psychology, Columbia University, New York 27, New York

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**Gutowsky, Herbert Sander,** 1960 (5), Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, Illinois

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**Hastings, Albert Baird,** 1939 (9), Scripps Clinic and Research Foundation, 476 Prospect Street, La Jolla, California

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**Heidelberger, Michael**, 1942 (10), Institute of Microbiology, Rutgers, The State University, New Brunswick, New Jersey

**Hendricks, Sterling Brown**, 1952 (5), Plant Industry Station, Beltsville, Maryland

**Herb, Raymond George**, 1955 (3), Department of Physics, University of Wisconsin, Madison 6, Wisconsin

**Hershey, Alfred Day**, 1958 (7), Department of Genetics, Carnegie Institution of Washington, Cold Spring Harbor, Long Island, New York

**Herskovits, Melville Jean**, 1959 (11), Department of Anthropology, Northwestern University, Evanston, Illinois

**Herzfeld, Karl Ferdinand**, 1960 (3), Department of Physics, Catholic University of America, Washington 17, D. C.

**Hess, Harry Hammond**, 1952 (6), Department of Geology, Princeton University, Princeton, New Jersey

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**Hildebrand, Joel Henry**, 1929 (5), Department of Chemistry, University of California, Berkeley 4, California

**Hilgard, Ernest Ropiequet**, 1948 (12), Department of Psychology, Stanford University, Stanford, California

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**Hollaender, Alexander**, 1957 (7), Biology Division, Oak Ridge National Laboratory, P. O. Box Y, Oak Ridge, Tennessee

**Holtfreter, Johannes**, 1955 (8), Biological Laboratories, University of Rochester, Rochester 3, New York

**Hoover, Herbert Clark**, 1922 (4), Waldorf Astoria Towers, New York, New York

**Hornig, Donald Frederick**, 1957 (5), Department of Chemistry, Princeton University, Princeton, New Jersey

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**Houston, William Vermillion**, 1943 (3), Rice University, Houston 1, Texas

**Hovland, Carl Iver**, 1960 (12), Department of Psychology, Yale University, 333 Cedar Street, New Haven 11, Connecticut

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**Hutchinson,** George **Evelyn,** 1950 (8), Osborn Zoological Laboratory, Yale University, New Haven 11, Connecticut

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**Johnson, William S**ummer, 1952 (5), Department of Chemistry and Chemical Engineering, Stanford University, Stanford, California

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**Panofsky, Wolfgang K. H.**, 1954 (3), High-Energy Physics Laboratory, Stanford University, Stanford, California

**Patterson, John Thomas**, 1941 (8), Department of Zoology, University of Texas, Austin 12, Texas

**Paul, John Rodman**, 1945 (10), Yale University School of Medicine, 333 Cedar Street, New Haven 11, Connecticut

**Pauling, Linus**, 1933 (5), Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena 4, California

**Pekeris, Chaim Leib**, 1952 (13), Department of Applied Mathematics, The Weizmann Institute of Science, Rehovot, Israel

**Petrunkevitch, Alexander**, 1954 (8), Osborn Zoological Laboratory, Yale University, New Haven 11, Connecticut

**Pfaffmann, Carl**, 1959 (12), Walter S. Hunter Laboratory of Psychology, Brown University, Providence 12, Rhode Island

**Pierce, John Robinson**, 1955 (4), Bell Telephone Laboratories, Incorporated, Murray Hill, New Jersey

**Piggot, Charles Snowden**, 1946 (13), Holly Grove, Lexington Park, Maryland

**Pitts, Robert Franklin**, 1956 (9), Cornell University Medical College, 1300 York Avenue, New York 21, New York

**Pitzer, Kenneth Sanborn**, 1949 (5), Department of Chemistry, University of California, Berkeley 4, California

**Press, Frank**, 1958 (13), Seismological Laboratory, California Institute of Technology, 220 North San Rafael Avenue, Pasadena, California

**Puck, Theodore Thomas**, 1960 (10), Department of Biophysics, University of Colorado Medical Center, Denver 20, Colorado

**Purcell, Edward Mills**, 1951 (3), Lyman Laboratory of Physics, Harvard University, Cambridge 38, Massachusetts

**Rabi, Isidor Isaac**, 1940 (3), Department of Physics, Columbia University, New York 27, New York

**Ramsey, Norman Foster**, 1952 (3), Lyman Laboratory of Physics, Harvard University, Cambridge 38, Massachusetts

**Raper, Kenneth Bryan**, 1949 (7), Department of Bacteriology, University of Wisconsin, Madison 6, Wisconsin

**Raymond, Arthur Emmons**, 1950 (4), 73 Oakmont Drive, Los Angeles 49, California

**Redfield, Alfred Clarence**, 1958 (9), Maury Lane, Woods Hole, Massachusetts

**Reichelderfer, Francis Wilton**, 1945 (13), United States Weather Bureau, Department of Commerce, Washington 25, D. C.

**Revelle, Roger Randall**, 1957 (13), University of California, La Jolla, La Jolla, California

**Rhoades, Marcus Morton**, 1946 (7), Department of Botany, Indiana University, Bloomington, Indiana

**Rich, Arnold Rice**, 1954 (10), Johns Hopkins Hospital, Baltimore 5, Maryland

**Richards, Alfred Newton**, 1927 (9), 737 Rugby Road, Bryn Mawr, Pennsylvania

**Richards, Dickinson W.**, 1958 (9), Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York 32, New York

**Richter, Curt Paul**, 1948 (12), Johns Hopkins Hospital, Baltimore 5, Maryland

**Riddle, Oscar**, 1939 (8), Route 4, Plant City, Florida

**Riker, Albert Joyce**, 1951 (7), Department of Plant Pathology, University of Wisconsin, Madison 6, Wisconsin

**Rittenberg, David**, 1953 (14), College of Physicians and Surgeons, 630 West 168th Street, New York 32, New York

**Rivers, Thomas Milton**, 1934 (10), Rockefeller Institute, New York 21, New York

**Robbins, William Jacob**, 1940 (7), 15 Dellwood Circle, Bronxville, New York

**Roberts, John D.**, 1956 (5), Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena 4, California

**Robertson, Howard Percy**, 1951 (3), California Institute of Technology, Pasadena, California

**Robertson, Oswald Hope**, 1943 (10), 9150 Los Gatos Highway, Santa Cruz, California

**Romer, Alfred Sherwood**, 1944 (8), Museum of Comparative Zoology at Harvard College, Oxford Street, Cambridge 38, Massachusetts

**Rose, William Cumming**, 1936 (14), University of Illinois, Urbana, Illinois

**Ross, Frank Elmore**, 1930 (2), 1100 Mt. Lowe Drive, Altadena, California

**Rossi, Bruno Benedetto**, 1950 (3), Room 26-569, Department of Physics, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

**Rossini, Frederick Dominic**, 1951 (5), After September 1, 1960: University of Notre Dame, Notre Dame, Indiana

**Rous, Francis Peyton**, 1927 (10), Rockefeller Institute, New York 21, New York

**Rubey, William Walden**, 1945 (6), United States Geological Survey, Department of the Interior, Washington 25, D. C.

**Russell, Richard Joel**, 1959 (6), Coastal Studies Institute, Louisiana State University, Baton Rouge 3, Louisiana

**Sabin, Albert Bruce**, 1951 (10), The Children's Hospital Research Foundation, University of Cincinnati College of Medicine, Elland Avenue and Bethesda, Cincinnati 29, Ohio

**Savage, John Lucian**, 1949 (4), 1651 Dahlia Street, Denver 20, Colorado

**Sax, Karl**, 1941 (7), Department of Botany, Josiah Willard Gibbs Research Laboratory, Yale University, New Haven, Connecticut. From September 1, 1960 to January 1, 1961: Department of Genetics, North Carolina State College, Raleigh, North Carolina

**Scatchard, George**, 1946 (5), Department of Chemistry, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

**Schairer, John Frank**, 1953 (6), Geophysical Laboratory, Carnegie Institution of Washington, 2801 Upton Street, N. W., Washington 8, D. C.

**Schiff, Leonard Isaac**, 1957 (3), Department of Physics, Stanford University, Stanford, California

**Schlesinger, Hermann Irving**, 1948 (5), Department of Chemistry, University of Chicago, Chicago 37, Illinois

**Schmidt, Carl Frederic**, 1949 (9), Laboratory of Pharmacology, School of Medicine, University of Pennsylvania, Philadelphia 4, Pennsylvania

**Schmitt, Francis Otto**, 1948 (8), Department of Biology, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

**Schrader, Franz**, 1951 (8), Department of Zoology, Duke University, Durham, North Carolina

**Schwarzschild, Martin**, 1956 (2), Princeton University Observatory, 14 Prospect Avenue, Princeton, New Jersey

**Schwinger, Julian**, 1949 (3), Department of Physics, Harvard University, Cambridge 38, Massachusetts

**Seaborg, Glenn Theodore**, 1948 (5), Lawrence Radiation Laboratory, University of California, Berkeley 4, California

**Seares, Frederick Hanley**, 1919 (2), El Encanto Hotel, Santa Barbara, California

**Segrè, Emilio**, 1952 (3), Department of Physics, University of California, Berkeley 4, California

**Seitz, Frederick**, 1951 (3), Department of Physics, University of Illinois, Urbana, Illinois

**Serber, Robert,** 1952 (3), Department of Physics, Columbia University, New York 27, New York

**Shaffer, Philip A**nderson, 1928 (14), School of Medicine, Washington University, Euclid Avenue and Kingshighway, St. Louis 10, Missouri

**Shannon, Claude E**lwood, 1956 (1), Research Laboratory of Electronics, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

**Shapiro, H**arry **L**ionel, 1949 (11), American Museum of Natural History, Central Park West at 79th Street, New York 24, New York

**Shapley, Harlow,** 1924 (2), Sharon Cross Road, Peterboro, New Hampshire

**Shedlovsky, Theodore,** 1953 (5), Rockefeller Institute, New York 21, New York

**Sheehan, John C**lark, 1957 (5), Department of Chemistry, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

**Shemin, David,** 1958 (14), Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York 32, New York

**Sherwood, Thomas K**ilgore, 1958 (4), Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

**Shockley, W**illiam, 1951 (4), Shockley Transistor Unit of Clevite Transistor, Stanford Industrial Park, Palto Alto, California

**Shope, Richard E**dwin, 1940 (10), Rockefeller Institute, New York 21, New York

**Simpson, G**eorge **G**aylord, 1941 (6), Museum of Comparative Zoology at Harvard College, Cambridge 38, Massachusetts

**Simpson, John A**lexander, 1959 (3), The Enrico Fermi Institute for Nuclear Studies, University of Chicago, Chicago 37, Illinois

**Sinnott, Edmund W**are, 1936 (7), Osborn Botanical Laboratory, Yale University, New Haven, Connecticut

**Skinner, B**urrhus **F**rederic, 1950 (12), Memorial Hall, Harvard University, Cambridge 38, Massachusetts

**Skoog, Folke** Karl, 1956 (7), Department of Botany, Birge Hall, University of Wisconsin, Madison 6, Wisconsin

**Slater, John C**larke, 1932 (3), Massachusetts Institute of Technology, Cambridge 39, Massachusetts

**Slepian, Joseph,** 1941 (4), 1115 Lancaster Street, Pittsburgh 18, Pennsylvania

**Slichter, Louis B**yrne, 1944 (13), Institute of Geophysics, University of California, Los Angeles 24, California

**Slipher, V**esto **M**elvin, 1921 (2), Lowell Observatory, Flagstaff, Arizona

**Smadel, Joseph E**dwin, 1957 (10), National Institutes of Health, Bethesda 14, Maryland

**Smith, Cyril Stanley,** 1957 (4), Institute for the Study of Metals, University of Chicago, Chicago 37, Illinois

**Smith, Homer W**illiam, 1945 (9), New York University School of Medicine, 550 First Avenue, New York 16, New York

**Smith, Lee Irvin,** 1944 (5), School of Chemistry, University of Minnesota, Minneapolis 14, Minnesota

**Smith, Paul A**lthaus, 1947 (1), Department of Mathematics, Columbia University, New York 27, New York

**Smith, Philip E**dward, 1939 (8), Department of Anatomy, Stanford University, Stanford, California

**Smyth, Charles P**helps, 1955 (5), Department of Chemistry, Princeton University, Princeton, New Jersey

**Snell, Esmond E**merson, 1955 (14), Department of Biochemistry, University of California, Berkeley 4, California

**Soderberg, C**arl **Richard,** 1947 (4), Massachusetts Institute of Technology, Cambridge 39, Massachusetts

**Sonneborn, T**racy **M**orton, 1946 (8), 220 Jordan Hall, Indiana University, Bloomington, Indiana

**Spedding, F**rank **H**arold, 1952 (5), Box 14-A, Station A, Institute for Atomic Research, Iowa State University, Ames, Iowa

**Spence, Kenneth W**artinbe, 1955 (12), Department of Psychology, State University of Iowa, Iowa City, Iowa

**Sperry, Roger W**olcott, 1960 (12), Division of Biology, California Institute of Technology, Pasadena, California

**Spier, Leslie,** 1946 (11), P. O. Box 880, Santa Cruz, California

**Spitzer, Lyman, Jr.,** 1952 (2), Princeton University Observatory, 14 Prospect Avenue, Princeton, New Jersey

**Stakman, E**lvin **C**harles, 1934 (7), Institute of Agriculture, University of Minnesota, St. Paul 1, Minnesota

**Stanley, Wendell M**eredith, 1941 (14), Virus Laboratory, University of California, Berkeley 4, California

**Stebbins, G**eorge **Ledyard,** 1952 (7), Department of Genetics, University of California, Davis, California

**Stebbins, Joel,** 1920 (2), 69 University Drive, Menlo Park, California

**Steenrod, N**orman **E**arl, 1956 (1), Box 708, Fine Hall, Princeton University, Princeton, New Jersey

**Stein, William H**oward, 1960 (14), Rockefeller Institute, New York 21, New York

**Stern, Curt,** 1948 (8), Department of Zoology, University of California, Berkeley 4, California

**Stern, Otto,** 1945 (3), 759 Cragmont Avenue, Berkeley 8, California

**Stevens, S**tanley **S**mith, 1946 (12), Memorial Hall, Harvard University, Cambridge 38, Massachusetts

**Steward, Julian H.,** 1954 (11), Department of Sociology and Anthropology, 137 Davenport Hall, University of Illinois, Urbana, Illinois

**Stockmayer, W**alter **H**ugo, 1956 (5), Department of Chemistry, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

**Stone, Marshall H**arvey, 1938 (1), 303 Eckhart Hall, University of Chicago, Chicago 37, Illinois

**Stone, Wilson S**tuart, 1960 (8), Genetics Foundation, University of Texas, Austin 12, Texas

**Stork, Gilbert** Josse, 1960 (5), Department of Chemistry, Columbia University, New York 27, New York

**Stratton, J**ulius **A**dams, 1950 (4), Massachusetts Institute of Technology, Cambridge 39, Massachusetts

**Street, J**abez **C**urry, 1953 (3), Jefferson Physical Laboratory, Harvard University, Cambridge 38, Massachusetts

**Struve, Otto,** 1937 (2), National Radio Astronomy Observatory, Green Bank, West Virginia

**Sturtevant, Alfred Henry**, 1930 (8), California Institute of Technology, Pasadena 4, California

**Suits, Chauncey Guy**, 1946 (4), General Electric Research Laboratory, The Knolls, Schenectady, New York

**Szent-Györgyi, Albert**, 1956 (14), Institute for Muscle Research, P. O. Box 187, Woods Hole, Massachusetts

**Taliaferro, William Hay**, 1940 (10), Argonne National Laboratory, 9700 South Cass Avenue, Argonne, Illinois

**Tarbell, Dean Stanley**, 1959 (5), Department of Chemistry, University of Rochester, Rochester 20, New York

**Tatum, Edward Lawrie**, 1952 (14), Rockefeller Institute, New York 21, New York

**Taube, Henry**, 1959 (5), Department of Chemistry, University of Chicago, Chicago 37, Illinois

**Teller, Edward**, 1948 (3), Lawrence Radiation Laboratory, University of California, P. O. Box 808, Livermore, California

**Terman, Frederick Emmons**, 1946 (4), Provost's Office, Stanford University, Stanford, California

**Thimann, Kenneth Vivian**, 1948 (7), The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge 38, Massachusetts

**Thomas, Charles Allen**, 1948 (4), Monsanto Chemical Company, 800 North Lindbergh Boulevard, St. Louis 66, Missouri

**Thomas, Llewellyn Hilleth**, 1958 (3), Watson Scientific Computing Laboratory, 612 West 116th Street, New York 27, New York

**Thomas, Tracy Yerkes**, 1941 (1), Graduate Institute for Mathematics and Mechanics, Indiana University, Bloomington, Indiana

**Thompson, Thomas Gordon**, 1951 (13), Department of Oceanography, University of Washington, Seattle 5, Washington

**Tillett, William Smith**, 1951 (10), New York University School of Medicine, 550 First Avenue, New York 16, New York

**Timoshenko, Stephen Prokop**, 1940 (4), 536 West Crescent, Palo Alto, California

**Tishler, Max**, 1953 (5), Merck & Co., Inc., Rahway, New Jersey

**Tousey, Richard**, 1960 (2), United States Naval Research Laboratory, Washington 25, D. C.

**Townes, Charles Hard**, 1956 (3), Department of Physics, Columbia University, New York 27, New York

**Turner, Francis John**, 1956 (6), Department of Geology, University of California, Berkeley 4, California

**Tuve, Merle Antony**, 1946 (3), Department of Terrestrial Magnetism, Carnegie Institution of Washington, 5241 Broad Branch Road, N. W., Washington 15, D. C.

**Twitty, Victor Chandler**, 1950 (8), Department of Biological Sciences, Stanford University, Stanford, California

**Uhlenbeck, George Eugene**, 1955 (3), Department of Physics, University of Michigan, Ann Arbor, Michigan

**Urey, Harold Clayton**, 1935 (5), University of California, La Jolla, California

**Van Allen, James Alfred**, 1959 (13), Department of Physics and Astronomy, State University of Iowa, Iowa City, Iowa

**Van Niel, Cornelis Bernardus**, 1945 (7), Hopkins Marine Station of Stanford University, Pacific Grove, California

**Van Slyke, Donald Dexter**, 1921 (14), Brookhaven National Laboratory, Upton, Long Island, New York

**Van Vleck, John Hasbrouck**, 1935 (3), Lyman Laboratory of Physics, Harvard University, Cambridge 38, Massachusetts

**Veblen, Oswald**, 1919 (1), The Institute for Advanced Study, Princeton, New Jersey

**Verhoogen, John**, 1956 (13), Department of Geology, University of California, Berkeley 4, California

**Vestine, Ernest Harry**, 1954 (13), Rand Corporation, 1700 Main Street, Santa Monica, California

**Vickery, Hubert Bradford**, 1943 (14), Connecticut Agricultural Experiment Station, New Haven 4, Connecticut

**Villard, Oswald Garrison, Jr.**, 1958 (13), Radioscience Laboratory, Stanford University, Stanford, California

**Visscher, Maurice Bolks**, 1956 (9), Department of Physiology, University of Minnesota, Minneapolis 14, Minnesota

von **Békésy, Georg**, 1956 (12), Psycho-Acoustic Laboratory, Memorial Hall, Harvard University, Cambridge 38, Massachusetts

**von Kármán, Theodore**, 1938 (4), 1501 South Marengo Avenue, Pasadena 5, California

**Waksman, Selman Abraham**, 1942 (10), Institute of Microbiology, Rutgers, The State University, New Brunswick, New Jersey

**Wald, George**, 1950 (14), The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge 38, Massachusetts

**Walker, John Charles**, 1945 (7), 206 Horticulture Building, University of Wisconsin, Madison 6, Wisconsin

**Walsh, Joseph Leonard**, 1936 (1), Widener 474, Harvard University, Cambridge 38, Massachusetts

**Warner, John Christian**, 1956 (5), Carnegie Institute of Technology, Pittsburgh 13, Pennsylvania

**Watson, Cecil James**, 1959 (10), Department of Medicine, University of Minnesota Hospital, Minneapolis 14, Minnesota

**Webster, David Locke**, 1923 (3), 1830 Cowper Street, Palo Alto, California

**Weiss, Paul Alfred**, 1947 (8), Rockefeller Institute, New York 21, New York

**Weisskopf, Victor Frederick**, 1952 (3), Department of Physics, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

**Went, Frits Warmolt**, 1947 (7), Missouri Botanical Garden, 2315 Tower Grove Avenue, St. Louis 10, Missouri

**Wentzel, Gregor**, 1959 (3), The Enrico Fermi Institute for Nuclear Studies, University of Chicago, Chicago 37, Illinois

**Werkman, Chester Hamlin**, 1946 (14), Department of Bacteriology, Iowa State University, Ames, Iowa

**Westheimer, Frank Henry**, 1954 (5), Department of Chemistry, Harvard University, 12 Oxford Street, Cambridge 38, Massachusetts

**Wetmore, Alexander,** 1945 (8), Smithsonian Institution, Washington 25, D. C.

**Wetmore, Ralph Hartley,** 1954 (7), The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge 38, Massachusetts

**Wever, Ernest Glen,** 1940 (12), 110 Eno Hall, Princeton University, Princeton, New Jersey

**Wheeler, John Archibald,** 1952 (3), Palmer Physical Laboratory, Princeton University, Princeton, New Jersey

**Whipple, Fred Lawrence,** 1959 (2), Astrophysical Observatory, Smithsonian Institution, 60 Garden Street, Cambridge 38, Massachusetts

**Whipple, George Hoyt,** 1929 (10), School of Medicine and Dentistry, University of Rochester, 260 Crittenden Boulevard, Rochester 20, New York

**Whitford, Albert Edward,** 1954 (2), Lick Observatory, Mt. Hamilton, California

**Whitney, Hassler,** 1945 (1), The Institute for Advanced Study, Princeton, New Jersey

**Whyburn, Gordon Thomas,** 1951 (1), Department of Mathematics, University of Virginia, Charlottesville, Virginia

**Wiesner, Jerome Bert,** 1960 (4), Research Laboratory of Electronics, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

**Wiggers, Carl John,** 1951 (9), 1360 Cleveland Heights Boulevard, Cleveland Heights 21, Ohio

**Wigner, Eugene Paul,** 1945 (3), 8 Ober Road, Princeton, New Jersey

**Willey, Gordon Randolph,** 1960 (11), Peabody Museum, Harvard University, Cambridge 38, Massachusetts

**Williams, Carroll Milton,** 1960 (8), The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge 38, Massachusetts

**Williams, Howel,** 1950 (6), Department of Geology, University of California, Berkeley 4, Calfornia

**Williams, John Warren,** 1952 (5), Department of Chemistry, University of Wisconsin, Madison 6, Wisconsin

**Williams, Robert R.,** 1945 (5), 297 Summit Avenue, Summit, New Jersey

**Williams, Robley Cook,** 1955 (14), Virus Laboratory, University of California, Berkeley 4, California

**Williams, Roger John,** 1946 (5), Clayton Foundation Biochemical Institute, University of Texas, Austin 12, Texas

**Willier, Benjamin Harrison,** 1945 (8), Department of Biology, Johns Hopkins University, Baltimore 18, Maryland

**Wilson, David Wright,** 1955 (14), Department of Biochemistry, School of Medicine, University of Pennsylvania, Philadelphia 4, Pennsylvania

**Wilson, Edgar Bright, Jr.,** 1947 (5), Department of Chemistry, Harvard University, 12 Oxford Street, Cambridge 38, Massachusetts

**Wilson, Edwin Bidwell,** 1919 (3), 42 Brington Road, Brookline 46, Massachusetts

**Wilson, Olin Chaddock,** 1960 (2), Mount Wilson and Palomar Observatories, 813 Santa Barbara Street, Pasadena, California

**Wilson, Perry William,** 1955 (7), Department of Bacteriology, University of Wisconsin, Madison 6, Wisconsin

**Wilson, Robert Erastus,** 1947 (4), U. S. Atomic Energy Commission, Washington 25, D. C.

**Wilson, Robert** Rathbun, 1957 (3), Laboratory of Nuclear Studies, Cornell University, Ithaca, New York

**Winstein, Saul,** 1955 (5), Department of Chemistry, University of California, Los Angeles 24, California

**Wintersteiner, Oskar,** 1950 (14), Squibb Institute for Medical Research, New Brunswick, New Jersey

**Wolfrom, M**elville **L**awrence, 1950 (5), Department of Chemistry, The Ohio State University, 121 West 19th Avenue, Columbus 10, Ohio

**Wood, Harland G**off, 1953 (14), Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland 6, Ohio

**Wood, W**illiam **Barry, Jr.,** 1959 (10), School of Medicine, Johns Hopkins University, 725 North Wolfe Street, Baltimore 5, Maryland

**Woodring, W**endell **P**hillips, 1946 (6), United States Geological Survey, Washington 25, D. C.

**Woodward, R**obert **B**urns, 1953 (5), Department of Chemistry, Harvard University, 12 Oxford Street, Cambridge 38, Massachusetts

**Woodworth, R**obert **S**essions, 1921 (12), 400 West 119th Street, New York 27, New York

**Woolley, D**ilworth **W**ayne, 1952 (14), Rockefeller Institute, New York 21, New York

**Woolsey, Clinton N**athan, 1960 (9), Laboratory of Neurophysiology, 219 Medical Sciences Building, University of Wisconsin, Madison 6, Wisconsin

**Wright, Sewall** Green, 1934 (8), Department of Genetics, University of Wisconsin, Madison 6, Wisconsin

**Wu, C**hien-**S**hiung, 1958 (3), Department of Physics, Columbia University, New York 27, New York

**Wulf, Oliver R**eynolds, 1949 (13), United States Weather Bureau, Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena 4, California

**Wyckoff, Ralph W**alter **G**raystone, 1949 (5), Departments of Bacteriology and Physics, University of Arizona, Tucson 25, Arizona

**Yoder, Hatten S**chuyler, **Jr.,** 1958 (6), Geophysical Laboratory, Carnegie Institution of Washington, 2801 Upton Street, N. W., Washington 8, D. C.

**Yost, Don M**erlin Lee, 1944 (5), California Institute of Technology, Pasadena 4, California

**Young, William G**ould, 1951 (5), Department of Chemistry, University of California, Los Angeles 24, California

**Zacharias, Jerrold R**einach, 1957 (3), Department of Physics, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

**Zachariasen,** Frederik **William H**oulder, 1949 (3), Division of the Physical Sciences, University of Chicago, Chicago 37, Illinois

**Zariski, Oscar,** 1944 (1), Department of Mathematics, Harvard University, 2 Divinity Avenue, Cambridge 38, Massachusetts

**Zener, Clarence** Melvin, 1959 (4), Westinghouse Research Laboratories, Beulah Road, Churchill Borough, Pittsburgh 35, Pennsylvania

**Zimm, Bruno H**asbrouck, 1958 (5), School of Science and Engineering, University of California, La Jolla, California

**Zinn, Walter Henry**, 1956 (4), Combustion Engineering, Inc., Nuclear Division, Windsor, Connecticut

**Zirkle, Raymond Elliott**, 1959 (8), Committee on Biophysics, University of Chicago, 5640 Ellis Avenue, Chicago 37, Illinois

**Zworykin, Vladimir Kosma**, 1943 (4), RCA Laboratories, David Sarnoff Research Center, Princeton, New Jersey

**Zygmund, Antoni**, 1960 (1), Department of Mathematics, University of Chicago, Chicago 37, Illinois

Number of Members July 1, 1960: 615.

## MEMBERS EMERITI

**Albright, Fuller**, 1952 (10), 271 Goddard Avenue, Brookline 46, Massachusetts

**Bailey, Irving Widmer**, 1929 (7), Harvard University Herbarium, 22 Divinity Avenue, Cambridge 38, Massachusetts

**Coble, Arthur Byron**, 1924 (1), Lykens Hotel, Lykens, Pennsylvania

**Coolidge, William David**, 1925 (3), 1480 Lenox Road, Schenectady 8, New York

**Goodpasture, Ernest William**, 1937 (10), 3407 Hopkins Lane, Nashville 12, Tennessee

**Hartman, Carl Gottfried**, 1937 (8), 219 Norwood Avenue, North Plainfield, New Jersey

**Mason, Max**, 1923 (3), 1035 Harvard Avenue, Claremont, California

**Saunders, Frederick Albert**, 1925 (3), South Hadley, Massachusetts

**Schultz, Adolph Hans**, 1939 (11), Anthropologisches Institut, Sempersteig 3, Zurich, Switzerland

**Tyzzer, Ernest Edward**, 1942 (10), 175 Water Street, Wakefield, Massachusetts

**Vandiver, Harry Shultz**, 1934 (1), Box 7881, University of Texas, Austin 12, Texas

Number of Members Emeriti July 1, 1960: 11.

## FOREIGN ASSOCIATES

The number in parentheses following the year of election indicates association within the sections of the National Academy of Sciences.

**Adrian of Cambridge**, Edgar Douglas, Baron, 1941 (9), Trinity College, Cambridge, England

**Alexandroff, Paul A.**, 1947 (1), Mathematical Institute of the Academy of Sciences of the U.S.S.R., Bolshaya Kalushskaya 19, Moscow, U.S.S.R.

**Ambartsumian, Victor Amazaspovich**, 1959 (2), Burakan Astronomical Observatory, Erevan, Armenia, U.S.S.R.

**Bailey, Sir Edward**, 1944 (6), 76 Hampstead Way, London, N. W. 11, England

**Bartlett, Sir Frederic Charles**, 1947 (12), 161 Huntingdon Road, Cambridge, England

**Best, Charles Herbert**, 1950 (9), Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada

**Bohr, Niels**, 1925 (3), Institute for Theoretical Physics, Blegdamsvej 15, Copenhagen, Denmark

**Bordet, Jules,** 1935 (10), Pasteur Institute, Rue du Remorqueur, 28, Brussels, Belgium

**Born, Max,** 1955 (3), Marcard Strasse 4, Bad Pyrmont, Germany

**Bragg, Sir** William **Lawrence,** 1945 (3), The Royal Institution, 21 Albemarle Street, London, W. 1, England

**de Broglie, Prince Louis,** 1948 (3), 94 Rue Perronet, Neuilly-sur-Siene, France

**Brun, Edmond** Antoine, 1960 (4), University of Paris, 8–10, place du Commerce, Paris XV, France

**Bullard, Sir Edward** Crisp, 1959 (13), Madingley Rise, Madingley Road, Cambridge, England

**Burnet, Sir Macfarlane,** 1954 (10), The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

**Caso, Alfonso,** 1943 (11), Av. Sudderman No. 312, Col. Chapultepec-Morales, Mexico 5, D. F.

**Chapman, Sydney,** 1946 (13), High Altitude Observatory, Boulder, Colorado, U. S. A.

**Dale, Sir Henry** Hallett, 1940 (9), The Wellcome Trust, 52 Queen Anne Street, London, W. 1, England

**Dirac, Paul** Adrien Maurice, 1949 (1), Department of Mathematics, St. John's College, Cambridge, England

**Eskola, Pentti** Eelis, 1951 (6), Helsinki University, Snellmanink. 5, Helsinki, Finland

**Fisher, Sir Ronald** Aylmer, 1948 (8), Division of Statistics, C.S.I.R.O., University of Adelaide, Adelaide, South Australia

**von Frisch, Karl,** 1951 (8), The Zoological Institute, University of Munich, Munich, Germany

**Geijer, Per,** 1958 (6), Agnevaegen 5, Djursholm I, Sweden

**Gregory, F**rederick **G**ugenheim, 1956 (7), Imperial College of Science and Technology, London, S.W. 7, England

**Hadamard, Jacques,** 1926 (1), 12 Rue Emile Faguet, Paris XIV, France

**Hartmann, Max,** 1959 (8), Max Planck Institute for Biology, Tübingen, Germany

**Hill, Archibald Vivian,** 1941 (9), 16 Bishopswood Road, Highgate, London, N.6, England

**Hinshelwood, Sir Cyril Norman,** 1960 (5), Department of Chemistry, Exeter College, Oxford, England

**Hodge,** William **V**allance **D**ouglas, 1959 (1), The Master's Lodge, Pembroke College, Cambridge, England

**Hopf, Heinz,** 1957 (1), Swiss Federal Institute of Technology, Alte Landstrasse 37, Zurich, Switzerland

**Houssay, Bernardo Alberto,** 1940 (9), Viamonte 2790, Buenos Aires, Argentina

**Jeffreys, Sir Harold,** 1945 (13), St. John's College, Cambridge, England

**Jones, Sir Harold Spencer,** 1943 (2), 40 Hesper Mews, London, S.W. 5, England

**Kapitza, Peter L**eonidovich, 1946 (3), S. I. Vavilov Institute of Physical Problems, Academy of Sciences of the U.S.S.R., Moscow, U.S.S.R.

**Karrer, Paul,** 1945 (5), University of Zurich, Zurich, Switzerland

**Kihara, H**itoshi, 1958 (7), National Institute of Genetics, Misima, Japan

**Krishnan, Sir Kariamanikkam** Srinivasa, 1956 (3), National Physical Laboratory of India, Hillside Road, New Delhi 12, India

**Landau, Lev Davidovich,** 1960 (3), S. I. Vavilov Institute of Physical Problems, Academy of Sciences of the U.S.S.R., Moscow, U.S.S.R.

**Leloir, Luis F.,** 1960 (14), Department of Biochemistry, University of Buenos Aires, Buenos Aires, Argentina

**Levi, Giuseppe,** 1940 (8), Instituto di Anatomia Umana, Corso Massimo D'Azeglio, 52, Turin, Italy

**Lim, Robert K. S.,** 1942 (9), Medical Sciences Research Laboratory, Miles Laboratories, Inc., Elkhart, Indiana, U. S. A.

**Lindblad, Bertil,** 1955 (2), Stockholm Observatory, Saltsjöbaden, Sweden

**Lwoff, André,** 1955 (9), Institut Pasteur, Paris XV, France

**Michotte, Albert** Edouard (Baron Michotte van den Berck), 1956 (12), University of Louvain, Louvain, Belgium

**Mott, N**evill **F**rancis, 1957 (3), University of Cambridge, Cavendish Laboratory, Free School Lane, Cambridge, England

**Oort, Jan** Hendrik, 1953 (2), Observatory of Leiden, Leiden, The Netherlands

**Penfield, Wilder,** 1953 (10), Montreal Neurological Institute, 3801 University Street, Montreal 2, Canada

**Pérès,** Joseph Jean Camille, 1956 (1), University of Paris, Paris V, France

**Piéron, Henri,** 1949 (12), Institute of Psychology, University of Paris, Paris, France

**Portevin, Albert** M. G. R., 1954 (4), 21, Boulevard de Beauséjour, Paris XVI, France

**Reichstein, Tadeus,** 1952 (5), Organisch-chemische Anstalt, St. Johanns-Ring 19, Basel, Switzerland

**Renner, Otto,** 1954 (7), The Botanical Institute, University of Munich, Munich, Germany

**Robinson, Sir Robert,** 1934 (5), 170 Piccadilly, London, W. 1, England

**Ruzicka, Leopold,** 1944 (5), Department of Organic Chemistry, Institute of Technology, Zurich, Switzerland

**Southwell, Sir Richard** Vynne, 1943 (4), The Old House, Trumpington, Cambridge, England

**Steacie, Edgar** William Richard, 1957 (5), National Research Council, Ottawa, Canada

**Svedberg, The,** 1945 (5), Fysikalisk-Kemiska Institutionen, Uppsala University, Uppsala, Sweden

**Taylor, Sir Geoffrey** Ingram, 1945 (1), Trinity College, Cambridge, England

**Theorell,** Axel **Hugo,** 1957 (14), Nobel Institute of Medicine, Solnavagen 1, Stockholm 60, Sweden

**Tiselius, Arne** W. K., 1949 (14), Institute of Biochemistry, Uppsala University, Uppsala, Sweden

**Todd, Sir Alexander** Robertus, 1955 (5), University of Cambridge, University Chemical Laboratory, Pembroke Street, Cambridge, England

**Vallée-Poussin, C. de la,** 1929 (1), 42, Avenue du Houx, Boitsfort, Belgium

**Vening Meinesz, F**elix **A**ndries, 1939 (13), Potgieterlaan 5, Amersfoort, The Netherlands

**Watson, D. M. S.,** 1938 (8), University College, Gower Street, London, W.C. 1, England

**Winge, Öjvind,** 1949 (14), Department of Physiology, Carlsberg Laboratory, Copenhagen (Valby), Denmark

**Yukawa, Hideki,** 1949 (3), Yukawa Hall, Kyoto University, Kyoto, Japan

Number of Foreign Associates July 1, 1960: 65.

## SECTIONS

### (1) *Mathematics*—36 members

Albert, A. A., *Chairman* (1961)
Ahlfors, L. V.
Alexander, J. W.
Artin, E.
Bell, E. T.
Bochner, S.
Brauer, R. D.
Courant, R.
Doob, J. L.
Douglas, Jesse
Eilenberg, S.
Eisenhart, L. P.
Evans, G. C.
Feller, William
Friedrichs, K. O.
Gödel, Kurt
Hille, Einar
Jacobson, Nathan
Lefschetz, Solomon
Mac Lane, Saunders
McShane, E. J.
Montgomery, Deane
Moore, R. L.
Morse, Marston
Murnaghan, F. D.
Shannon, C. E.
Smith, Paul A.
Steenrod, N. E.
Stone, M. H.
Thomas, T. Y.
Veblen, Oswald
Walsh, J. L.
Whitney, Hassler
Whyburn, G. T.
Zariski, O.
Zygmund, Antoni

*Foreign Associates*

Alexandroff, P. A.
Dirac, P. A. M.
Hadamard, Jacques
Hodge, W. V. D.
Hopf, Heinz
Pérès, Joseph
Taylor, Sir Geoffrey
Vallée-Poussin, C. de la

### (2) *Astronomy*—30 members

Mayall, N. U., *Chairman* (1962)
Abbot, C. G.
Babcock, H. D.
Babcock, H. W.
Bowen, I. S.
Brouwer, Dirk
Chandrasekhar, S.
Clemence, G. M.
Goldberg, Leo
Greenstein, J. L.
Joy, A. H.
Kuiper, G. P.
McMath, R. R.
Menzel, D. H.
Merrill, P. W.
Minkowski, R. L.
Morgan, W. W.
Nicholson, S. B.
Ross, F. E.
Schwarzschild, M.
Seares, F. H.
Shapley, Harlow
Slipher, V. M.
Spitzer, Lyman, Jr.
Stebbins, Joel
Struve, Otto
Tousey, Richard
Whipple, F. L.
Whitford, A. E.
Wilson, Olin C.

*Foreign Associates*

Ambartsumian, V.
Jones, Sir H. Spencer
Lindblad, Bertil
Oort, Jan Hendrik

### (3) *Physics*—92 members

Allison, S. K., *Chairman* (1963)
Alvarez, L. W.
Anderson, C. D.
Anderson, H. L.
Bacher, R. F.
Bainbridge, K. T.
Bardeen, John
Beams, J. W.

Bethe, H. A.
Birge, R. T.
Bleakney, Walker
Bloch, Felix
Bloembergen, N.
Bonner, T. W.
Bradbury, N. E.
Brattain, W. H.
Breit, Gregory
Bridgman, P. W.
Brillouin, Leon
Brode, R. B.
Chamberlain, Owen
Coblentz, W. W.
Compton, A. H.
Condon, E. U.
Dennison, D. M.
Deutsch, M.
DuBridge, L. A.
DuMond, J. W. M.
Dunning, J. R.
Elsasser, W. M.
Epstein, P. S.
Feynman, R. P.
Fowler, W. A.
Franck, James
Gamow, George
Gell-Mann, Murray
Goldhaber, M.
Goudsmit, S. A.
Herb, R. G.
Herzfeld, K. F.
Hofstadter, R.
Houston, W. V.
Hull, A. W.
Kemble, E. C.
Kent, R. H.
Kerst, D. W.
Kittel, C.
Kusch, P.
Lamb, W E., Jr.
Land, E. H.
Lauritsen, C. C.
Loomis, F. W.
Marshak, R. E.
Mayer, M. G.
McMillan, E. M.
Meggers, W. F.
Morse, P. M.
Mulliken, R. S.
Nier, A. O. C.
O'Brien, Brian
Oppenheimer, J. R.
Panofsky, W. K. H.
Purcell, E. M.
Rabi, I. I.
Ramsey, N. F.
Robertson, H. P.
Rossi, Bruno
Schiff, L. I.
Schwinger, Julian
Segrè, E.
Seitz, Frederick
Serber, R.
Simpson, J. A.
Slater, J. C.
Stern, Otto
Street, J. C.
Teller, Edward
Thomas, L. H.
Townes, C. H.
Tuve, M. A.
Uhlenbeck, G. E.
Van Vleck, J. H.
Webster, D. L.
Weisskopf, V. F.
Wentzel, Gregor
Wheeler, J. A.
Wigner, E. P.
Wilson, Edwin B.
Wilson, Robert R.
Wu, C. S.
Zacharias, J. R.
Zachariasen, W. H.

*Foreign Associates*

Bohr, Niels
Born, Max
Bragg, Sir Lawrence
de Broglie, Prince Louis
Kapitza, P. L.
Krishnan, Sir K.
Landau, L. D.
Mott, N. F.
Yukawa, Hideki

### (4) *Engineering*—46 members

Fisk, J. B., *Chairman* (1962)
Astin, A. V.
Bain, E. C.
Benedict, Manson
Bode, H. W.
Briggs, L. J.
Bush, Vannevar
Chipman, John
Curme, G. O., Jr.
Den Hartog, J. P.
Draper, C. S.
Dryden, H. L.
Fletcher, Harvey
Foote, P. D.
Gibbs, W. F.
Gilliland, E. R.
Greenewalt, C. H.
Hoover, Herbert
Hunsaker, J. C.
Jeffries, Zay
Kelly, M. J.
Kinzel, A. B.
Lewis, W. K.
Loomis, A. L.
Mehl, R. F.
Murphree, E. V.
Olson, Harry F.
Pierce, J. R.
Raymond, A. E.
Savage, J. L.
Sherwood, T. K.
Shockley, W.
Slepian, Joseph
Smith, C. S.
Soderberg, C. R.
Stratton, J. A.
Suits, C. G.
Terman, F. E.
Thomas, C. A.
Timoshenko, Stephen
von Kármán, T.

Wiesner, J. B.
Wilson, Robert E.
Zener, Clarence
Zinn, W. H.
Zworykin, V. K.

*Foreign Associates*

Brun, Edmond A.
Portevin, Albert M. G. R
Southwell, Sir Richard V.

### (5) *Chemistry*—90 members

Pitzer, K. S., *Chairman* (1962)
Adams, Roger
Badger, R. M.
Bartlett, P. D.
Blomquist, A. T.
Bolton, E. K.
Brewer, Leo
Brode, W. R.
Brown, H. C.
Calvin, Melvin
Conant, J. B.
Cope, A. C.
Crawford, Bryce, Jr.
Daniels, Farrington
Davidson, N.
Debye, Peter
Doty, Paul
Elderfield, R. C.
Emmett, P. H.
Eyring, Henry
Ferry, John D.
Fieser, L. F.
Flory, P. J.
Folkers, Karl
Fuoss, R. M.
Fuson, R. C.
Gates, M.
Giauque, W. F.
Gilman, Henry
Gutowsky, H. S.
Hammett, L. P.
Harned, H. S.
Hauser, C. R.
Hendricks, S. B.
Hildebrand, J. H.
Hirschfelder, J. O.
Hornig, D. F.
Jacobs, W. A.
Johnson, J. R.
Johnson, W. S.
Keyes, F. G.
Kimball, G. E.
Kistiakowsky, G. B.
Kolthoff, I. M.
Kraus, C. A.
La Mer, V. K.
Leonard, N. J.
Libby, W. F.
Lind, S. C.
Longsworth, L. G.
Lucas, H. J.
MacInnes, D. A.
Marvel, C. S.
Mayer, J. E.
McElvain, S. M.
Mees, C. E. K.
Newman, M. S.
Niemann, C. G.
Noyes, W. A., Jr.
Onsager, Lars
Pauling, Linus
Roberts, John D.
Rossini, F. D.
Scatchard, George
Schlesinger, H. I.
Seaborg, G. T.
Shedlovsky, Theodore
Sheehan, J. C.
Smith, L. I.
Smyth, C. P.
Spedding, F. H.
Stockmayer, W. H.
Stork, Gilbert
Tarbell, D. S.
Taube, Henry
Tishler, Max
Urey, H. C.
Warner, J. C.
Westheimer, F. H.
Williams, J. W.
Williams, Robert R.
Williams, Roger J.
Wilson, E. Bright, Jr.
Winstein, Saul
Wolfrom, M. L.
Woodward, R. B.
Wyckoff, R. W. G.
Yost, D. M.
Young, W. G.
Zimm, B. H.

*Foreign Associates*

Hinshelwood, Sir C.N.
Karrer, Paul
Reichstein, Tadeus
Robinson, Sir Robert
Ruzicka, Leopold
Steacie, E. W. R.
Svedberg, The
Todd, Sir Alexander

### (6) *Geology*—34 members

Hess, H. H., *Chairman* (1963)
Abelson, P. H.
Allen, E. T.
Anderson, C. A.
Birch, Francis
Blackwelder, Eliot
Bradley, W. H.
Bramlette, M. N.

Bucher, W. H.
Buddington, A. F.
Buerger, M. J.
Chaney, R. W.
Cloos, Ernst
Dunbar, C. O.
Gilluly, James
Gregory, W. K.
Hedberg, H. D.
Hewett, D. F.
Hubbert, M. King
Kelley, W. P.
Knopf, Adolph
Krauskopf, K. B.
Larsen, E. S., Jr.
Longwell, C. R.
Lovering, T. S.
Nolan, T. B.
Rubey, W. W.
Russell, R. J.
Schairer, J. F.
Simpson, G. G.
Turner, F. J.
Williams, Howel
Woodring, W. P.
Yoder, H. S., Jr.

*Foreign Associates*

Bailey, Sir Edward
Eskola, Pentti
Geijer, Per

### (7) *Botany*—43 members

Riker, A. J., *Chairman* (1962)
Anderson, Edgar
Barker, H. A.
Beadle, G. W.
Blinks, L. R.
Bonner, David M.
Bonner, James
Braun, A. C.
Brink, R. A.
Burkholder, P. R.
Chandler, W. H.
Clausen, Jens
Cleland, R. E.
Couch, J. N.
Delbrück, Max
Dodge, B. O.
Esau, Katherine
Fred, E. B.
Goddard, D. R.
Hershey, A. D.
Hollaender, A.
Horsfall, J. G.
Jones, D. F.
Kaufmann, B. P.
Lederberg, J.
Luria, S. E.
Mangelsdorf, P. C.
McClintock, Barbara
Osterhout, W. J. V.
Raper, K. B.
Rhoades, M. M.
Robbins, W. J.
Sax, Karl
Sinnott, E. W.
Skoog, Folke
Stakman, E. C.
Stebbins, G. L.
Thimann, K. V.
Van Niel, C. B.
Walker, J. C.
Went, F. W.
Wetmore, R. H.
Wilson, P. W.

*Foreign Associates*

Gregory, F. G.
Kihara, H.
Renner, Otto

### (8) *Zoology and Anatomy*—53 members

Demerec, Milislav, *Chairman* (1961)
Bartelmez, G. W.
Bigelow, H. B.
Bloom, William
Bodenstein, D. H.
Bodian, David
Burns, R. K.
Castle, W. E.
Cleveland, L. R.
Colbert, E. H.
Corner, G. W.
Danforth, C. H.
Dobzhansky, Th.
Dunn, L. C.
Glass, Bentley
Griffin, D. R.
Hamburger, Viktor
Haskins, C. P.
Hisaw, F. L.
Holtfreter, J.
Hubbs, C. L.
Hutchinson, G. E.
Irwin, M. R.
Jacobs, M. H.
Kellogg, Remington
Lerner, I. M.
Lewis, W. H.
Mayr, Ernst
Mazia, Daniel

Metz, C. W.
Miller, A. H.
Mirsky, A. E.
Muller, H. J.
Nicholas, J. S.
Painter, T. S.
Patterson, J. T.
Petrunkevitch, A.
Riddle, Oscar
Romer, A. S.
Schmitt, F. O.
Schrader, Franz
Smith, Philip E.
Sonneborn, T. M.
Stern, Curt
Stone, W. S.
Sturtevant, A. H.
Twitty, V. C.
Weiss, Paul
Wetmore, Alexander
Williams, C. M.
Willier, B. H.
Wright, Sewall
Zirkle, R. E.

*Foreign Associates*

Fisher, Sir Ronald
von Frisch, Karl
Hartmann, Max
Levi, Giuseppe
Watson, D. M. S.

### (9) *Physiology*—34 members

Long, C. N. H., *Chairman* (1963)
Astwood, E. B.
Aub, Joseph C.
Bard, Philip
Brink, Frank, Jr.
Bronk, Detlev W.
Cole, K. S.
Cournand, André
Davis, Hallowell
Erlanger, Joseph
Evans, H. M.
Fenn, W. O.
Forbes, Alexander
Gasser, H. S.
Gerard, R. W.
Hartline, H. K.
Hastings, A. B.
Landis, E. M.
Lloyd, D. P. C.
Loeb, R. F.
Lorente de Nó, R.
Magoun, H. W.
Mann, Frank
Marshall, E. K., Jr.
Meek, W. J.
Pitts, R. F.
Redfield, A. C.
Richards, A. N.
Richards, D. W.
Schmidt, C. F.
Smith, Homer W.
Visscher, M. B.
Wiggers, C. J.
Woolsey, C. N.

*Foreign Associates*

Lord Adrian
Best, C. H.
Dale, Sir Henry
Hill, A. V.
Houssay, B. A.
Lim, Robert K. S.
Lwoff, André

### (10) *Pathology and Microbiology*—42 members

Shope, R. E., *Chairman* (1963)
Armstrong, Charles
Bailey, P.
Blalock, Alfred
Cannon, P. R.
Castle, W. B.
Coggeshall, L. T.
Cole, Rufus
Dalldorf, Gilbert
Dingle, J. H.
Dochez, A. R.
Dragstedt, L. R.
Dubos, R. J.
Enders, J. F.
Francis, Thomas, Jr.
Goebel, W. F.
Heidelberger, Michael
Horsfall, F. L., Jr.
Huebner, R. J.
Huggins, C. B.
Little, C. C.
Long, E. R.
MacLeod, C. M.
Maxcy, K. F.
McMaster, P. D.
Meyer, K. F.
Miller, C. P.
Opie, E. L.
Paul, John R.
Puck, T. T.
Rich, A. R.
Rivers, T. M.
Robertson, O. H.
Rous, Peyton
Sabin, Albert B.
Smadel, J. E.
Taliaferro, W. H.
Tillett, W. S.
Waksman, S. A.
Watson, C. J.
Whipple, G. H.
Wood, W. Barry, Jr.

*Foreign Associates*

Bordet, Jules
Burnet, Sir Macfarlane
Penfield, Wilder

### (11) *Anthropology*—13 members

Haury, Emil W. *Chairman* (1963)
Albright, W. F.
Coon, C. S.
Herskovits, M. J.
Kidder, A. V.
Kluckhohn, Clyde
Kroeber, A. L.
Lothrop, S. K.
Movius, H. L., Jr.
Shapiro, H. L.
Spier, Leslie
Steward, J. H.
Willey, G. R.

*Foreign Associate*

Caso, Alfonso

### (12) *Psychology*—24 members

Lindsley, D. B., *Chairman* (1962)
Beach, F. A.
Boring, E. G.
Carmichael, Leonard
Galambos, R.
Gesell, Arnold
Graham, C. H.
Guilford, J. P.
Harlow, H. F.
Hilgard, E. R.
Hovland, C. I.
Klüver, Heinrich
Köhler, Wolfgang
Miles, W. R.
Miller, N. E.
Pfaffmann, Carl
Richter, C. P.
Skinner, B. F.
Spence, K. W.
Sperry, R. W.
Stevens, S. S.
v. Békésy, Georg
Wever, E. G.
Woodworth, R. S.

*Foreign Associates*

Bartlett, Sir Frederic
Michotte, Albert E.
Piéron, Henri

### (13) *Geophysics*—30 members

Slichter, L. B., *Chairman* (1963)
Adams, L. H.
Benioff, Hugo
Berkner, L. V.
Bjerknes, J.
Booker, H. G.
Brown, Harrison
Byerly, Perry
Byers, H. R.
Eckart, Carl
Ewing, Maurice
Friedman, H.
Griggs, D. T.
Gunn, Ross
Haurwitz, B.
Iselin, C. O'D.
Kaplan, Joseph
Lambert, W. D.
Munk, W. H.
Pekeris, C. L.
Piggot, C. S.
Press, Frank
Reichelderfer, F. W.
Revelle, Roger
Thompson, T. G.
Van Allen, J. A.
Verhoogen, John
Vestine, E. H.
Villard, O. G., Jr.
Wulf, O. R.

*Foreign Associates*

Bullard, Sir Edward
Chapman, Sydney
Jeffreys, Sir Harold
Vening Meinesz, F. A.

## (14) *Biochemistry*—48 members

du Vigneaud, V., *Chairman* (1961)
Anderson, R. J.
Ball, E. G.
Balls, A. K.
Bloch, K. E.
Carter, H. E.
Chance, Britton
Clark, W. M.
Clarke, H. T.
Cori, Carl F.
Craig, L. C.
Doisy, E. A.
Edsall, J. T.
Elvehjem, C. A.
Fruton, J. S.
Hassid, W. Z.
Kalckar, H. M.
Kendall, E. C.
King, C. G.
Kornberg, A.
Lardy, Henry
Lehninger, A. L.
Link, K. P.
Lipmann, Fritz
Maynard, L. A.
McCollum, E. V.
Moore, Stanford
Northrop, J. H.
Ochoa, Severo
Oncley, J. L.
Rittenberg, David
Rose, W. C.
Shaffer, P. A.
Shemin, David
Snell, E. E.
Stanley, W. M.
Stein, W. H.
Szent-Györgyi, A.
Tatum, E. L.
Van Slyke, D. D.
Vickery, H. B.
Wald, George
Werkman, C. H.
Williams, R. C.
Wilson, D. W.
Wintersteiner, O.
Wood, H. G.
Woolley, D. W.

*Foreign Associates*

Leloir, Luis F.
Theorell, Hugo
Tiselius, Arne W. K.
Winge, Öjvind

## GEOGRAPHICAL LISTING of ACADEMY MEMBERS

### *Arizona*

Haury, Emil W.
Kuiper, G. P.
Slipher, V. M.
Wyckoff, R. W. G.

### *California*

Adams, L. H.
Alvarez, L. W.
Anderson, C. D.
Babcock, H. D.
Babcock, H. W.
Bacher, R. F.
Badger, R. M.
Balls, A. K.
Barker, H. A.
Beach, F. A.
Beadle, G. W.
Bell, E. T.
Benioff, Hugo
Birge, R. T.
Bjerknes, J.
Blackwelder, Eliot
Blinks, L. R.
Bloch, Felix
Bonner, James
Bowen, I. S.
Bramlette, M. N.
Brewer, Leo
Brode, R. B.
Brown, Harrison
Byerly, Perry
Calvin, Melvin
Castle, W. E.
Chamberlain, Owen
Chandler, W. H.
Chaney, R. W.
Clausen, Jens
Danforth, C. H.
Davidson, N.
Delbrück, Max
DuBridge, L. A.
DuMond, J. W. M.
Eckart, Carl
Elsasser, W. M.
Epstein, P. S.
Esau, Katherine
Evans, G. C.
Evans, H. M.
Feynman, R. P.
Fowler, W. A.
Gell-Mann, Murray
Giauque, W. F.
Greenstein, J. L.
Griggs, D. T.
Guilford, J. P.
Hassid, W. Z.
Hastings, A. Baird
Hewett, D. F.
Hildebrand, J. H.
Hilgard, E. R.
Hofstadter, R.
Hubbs, C. L.
Johnson, W. S.
Joy, A. H.
Kaplan, Joseph
Kelley, W. P.
Kerst, D. W.
Kittel, C.
Knopf, Adolph
Kornberg, A.
Krauskopf, K. B.
Kroeber, A. L.
Lauritsen, C. C.
Lederberg, J.
Lerner, I. M.
Libby, W. F.
Lindsley, D. B.
Longwell, C. R.
Lucas, H. J.
Magoun, H. W.
Mason, Max
Mayall, N. U.
Mayer, J. E.
Mayer, M. G.
Mazia, Daniel
McMillan, E. M.
Merrill, P. W.
Meyer, K. F.
Miller, A. H.
Minkowski, R. L.
Munk, W. H.
Nicholson, S. B.
Niemann, Carl
Northrop, J. H.
Panofsky, W. K. H.
Pauling, Linus
Pitzer, K. S.
Press, Frank
Raymond, A. E.
Revelle, Roger
Roberts, John D.
Robertson, H. P.
Robertson, O. H.
Ross, F. E.
Schiff, L. I.
Seaborg, G. T.
Seares, F. H.
Segrè, Emilio
Shockley, W.
Slichter, L. B.
Smith, P. E.
Snell, E. E.
Sperry, R. W.
Spier, Leslie
Stanley, W. M.
Stebbins, G. L.
Stebbins, Joel
Stern, Curt
Stern, Otto
Sturtevant, A. H.
Teller, Edward
Terman, F. E.
Timoshenko, S. P.
Turner, F. J.
Twitty, V. C.
Urey, H. C.
Van Niel, C. B.
Verhoogen, John
Vestine, E. H.
Villard, O. G., Jr.
von Kármán, T.
Webster, D. L.
Whitford, A. E.
Williams, Howel
Williams, R. C.
Wilson, Olin C.
Winstein, Saul
Wulf, O. R.
Yost, D. M.
Young, W. G.
Zimm, B. H.

### *Colorado*

*Chapman, Sydney
Gamow, George
Gilluly, James

* Foreign Associate

Haurwitz, B.
Lovering, T. S.
Puck, T. T.
Savage, J. L.

### *Connecticut*

Anderson, R. J.
Bonner, David M.
Breit, G.
Brouwer, Dirk
Clarke, H. T.
Dunbar, C. O.
Fruton, J. S.
Fuoss, R. M.
Gesell, Arnold
Harned, H. S.
Hille, Einar
Horsfall, J. G.
Hovland, C. I.
Hutchinson, G. E.
Jacobson, Nathan
Jones, D. F.
Lambert, W. D.
Long, C. N. H.
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